ENVIRONMENTAL, SOCIAL, AND GENETIC FACTORS PREDISPOSING *XENOPUS LAEVIS* TADPOLES TO INFECTION

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By

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ABSTRACT

This work examines the ecological, social and genetic factors that predispose amphibians to infection. In the last 30 years many amphibian populations have declined due to infectious disease, although few researchers have studied the factors involved in mediating amphibian infection. My research is designed to explore some of these factors.

I first examined the effects of crowding, kin composition (the relatedness of individuals in a group), and habitat complexity on the growth and survival of *Xenopus laevis* tadpoles exposed to the bacterial pathogen *Aeromonas hydrophila*. In tadpoles, stress, and in particular corticosterone, a hormone associated with stress, is known to inhibit growth. Crowding, kin composition, and habitat complexity have all been linked to tadpole growth. As corticosterone exposure is also linked to reduced immune function, I examined how these ecological factors influence tadpoles' disease resistance. Tadpoles exposed to the bacterium were significantly smaller and more likely to die than control tadpoles. Tadpoles reared only with siblings (pure sibship groups) were larger, less variable in size, and had lower mortality rates than tadpoles reared in mixed sibship groups. The size difference between pure and mixed sibship groups was greatest when they were exposed to the pathogen. Habitat complexity reduced size variation within tanks but did not affect mean tadpole size. Mixed kinship composition and high tadpole density can increase competition, reduce growth, and increase disease susceptibility. These results indicate that growth was inhibited by pathogen exposure but kin association may ameliorate this effect.

The Major Histocompatibility Complex (MHC) is an integral part of the vertebrate adaptive immune system. To determine the importance of the MHC in conferring disease resistance in amphibians, I challenged *X. laevis* tadpoles, bearing different combinations of four MHC haplotypes (*f*, *g*, *j*, and *r*), with *A. hydrophila* in two experiments. Exposure to *A. hydrophila* affected the growth and survival of these tadpoles and that the MHC moderated these effects. Tadpoles with two MHC haplotypes (*r* and *g*) were susceptible to this pathogen and tadpoles with the other two haplotypes (*f* and *j*) were resistant. Heterozygous tadpoles with both susceptible and resistant haplotypes were always intermediate to either homozygotes in size and survival. These results demonstrate that MHC genotype plays a major role in determining the impact of bacterial pathogens on the growth and survival of *X. laevis* tadpoles.

To test whether the effect of exposure to pathogens differs according to the similarity of the hosts I challenged tadpoles with natural levels of the microorganisms associated with different MHC genotypes by exposing the tadpoles to water preconditioned by adults of different MHC genotypes. If the pathogens are adapted to the MHC genotype of their hosts, tadpoles exposed to water from adults with which they shared MHC haplotypes would be more susceptible than those exposed to water from MHC-dissimilar adults. Alternatively, if the hosts are adapted to their pathogens tadpoles may be more resistant to pathogens from MHC-similar frogs than those from MHC-dissimilar frogs. I found that tadpoles exposed to water from MHC-dissimilar animals developed faster, but without increased growth, and

were more likely to die than those exposed to water from MHC-similar animals. Furthermore, there was an optimal difference between the tadpoles' and the donors' MHC where tadpoles were sufficiently different to the donor to defend against its locally adapted pathogens, and sufficiently similar to not be exposed to especially virulent foreign pathogens.

Finally, I present an inventory of bacteria found in the gut and skin (non-systemic sites) and heart, muscle, and abdominal cavity (systemic sites) of captive frogs. I found several species of bacteria previously identified as amphibian pathogens and many bacteria in systemic sites that have not been considered pathogenic to amphibians. None of the frogs tested positive for the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. I discuss the potential importance of these species of bacteria as amphibian pathogens and as protective probiotics, using New Zealand frogs as a case study.

In its sum, this work describes some of the factors that can affect amphibians' ability to resist disease. I show that the genetic constitution of an individual, specifically in terms of the MHC, affects the impact of a disease, and so too does its social and ecological conditions, including the level of crowding, the kinship of its groupmates and the specific microbial challenges of its immediate environment. I also show that many of the factors linked to tadpole growth and development that are well described in other amphibians also affect *Xenopus* tadpoles.

CHAPTER 1: INTRODUCTION

Amphibians have been declining worldwide at an alarming rate and while pathogens have been implicated in these declines few studies have attempted to determine the factors that predispose amphibians to disease. The aim of this thesis is to describe some of the ecological, social, and genetic factors that affect amphibians' ability to deal with pathogens in their environment.

Pathogen Driven Amphibian Declines

The drastic declines of amphibian populations, including species and local extinctions have been documented with alarming regularity over the past thirty years (Burrowes *et al.* 2004; Carey 1997, 2000; Carey *et al.* 1999; Daszak *et al.* 1999, 2003; Houlahan *et al.* 2000; Lips *et al.* 2006). These declines have been widely attributed to outbreaks of disease by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Berger *et al.* 1998; Bosch *et al.* 2001; Burrowes *et al.* 2004; Lips *et al.* 2006, 2004; Taylor *et al.* 1999b), although other infectious agents have been implicated in amphibian die-offs, such as *Aeromonas hydrophila* (Bradford 1991; Frye 1985; Nyman 1986) and iridoviruses (Cunningham *et al.* 1996; Green *et al.* 2002). The impact of *B. dendrobatidis* is remarkable in two major ways.

First, the scale of the amphibian chytrid fungus is unprecedented. Amphibian declines have been documented in all six continents that amphibians inhabit, and the amphibian chytrid fungus is associated with the declines of 93 species worldwide (Daszak *et al.* 2001; Lips *et al.* 2006; Schoegel *et al.* 2005). It appears that *B. dendrobatidis* is a recently emerged pathogen that has been introduced into naive

populations (Cunningham *et al.* 2003; Daszak *et al.* 2003; Lips *et al.* 2006; McDonald *et al.* 2005; Morehouse *et al.* 2003; Retallick *et al.* 2004) with disastrous consequences. It is encouraging to know that survivors of chytrid induced epizootics can persist with the pathogen, and these populations may yet recover (Retallick *et al.* 2004). The long-term survival of these genetically impoverished groups is uncertain. The reduction in genetic diversity of these populations could increase the incidence of inbreeding and its associated fitness effects, including greater susceptibility to other diseases (Acevedo-Whitehouse *et al.* 2003, 2005; Coltman *et al.* 1999; Patenaude *et al.* 1994; Pearman & Garner 2005; Waldman & Tocher 1998). Indeed, if *B. dendrobatidis* recently evolved into its current, highly virulent form, further mutations may endanger the remaining individuals, populations, or species resistant to the current strain.

Second, almost all conventional models of host-pathogen dynamics suggest that pathogens should rarely drive hosts to extinction or even to become critically endangered (Smith *et al.* 2006). Since pathogen transmission is linked to host density, the pathogens are predicted to become extinct before their hosts. Nonetheless, small localized populations with limited genetic variability and the presence of reservoir host species may become extinct as a result of infectious organisms (de Castro & Bolker 2005; McCallum & Dobson 1995). Smith *et al.* (2006) supported the contention that diseases are rarely responsible for extinctions or driving species to critically endangered status. They found that pathogens account for less than 4% of all extinctions since the 19th century, and were responsible for under 8% of the critically endangered species, although amphibians were strongly overrepresented in this study, accounting for 75% of the critically endangered

species threatened by disease.

The maintenance of diversity in the major histocompatibility complex (MHC) may be especially important in maintaining the viability of amphibian populations, as this gene complex is responsible for the detection of diseases. Thus, diminished MHC variation may leave a population prone to possibly devastating further outbreaks. Individuals that have survived an outbreak and share the same MHC sequences are also likely to be related, and may then be forced to breed with close kin, which in turn produces further genetic homozygosity, and inbreeding depression, both of which reduce fitness and may lead to a local extinction if further negative events occur.

In this thesis I sought to expand the understanding of what predisposes amphibians to disease. While the amphibian chytrid fungus has been convincingly demonstrated to be the etiological agent in a number of declines (i.e. Lips *et al.* 2006), it is unclear why some populations are susceptible while others are not, and what factors might increase the impacts of disease in amphibians. I explored some of the ecological and genetic factors that could affect amphibians' resistance to a pathogen using African clawed frog (*Xenopus laevis*) tadpoles as a model host, and the ubiquitous amphibian bacterial pathogen *A. hydrophila* as a model pathogen.

The Ecology of Disease Resistance

Unknown environmental stressors may have played a role in amphibian disease and possibly declines by inhibiting immune function. Crowding is well known to inhibit tadpole growth and development (Adolf 1931; Brockelman 1969; Dash & Hota 1980; Gromko *et al.* 1973; Hota & Dash 1981; Licht 1967; Lynn & Edelman 1936; Murray

1990; Richards 1958; Semlitsch & Caldwell 1982; Warner *et al.* 1991; Wilbur 1976; Wilbur 1977), and is linked to increased corticosterone levels (Glennemeir & Denver 2002) and to increased disease susceptibility in other taxa (Goulson & Cory 1995; Pai *et al.* 1995; Wedemeyer 1997). Stress hormones such as corticosterone can inhibit both adaptive and innate immune function in amphibians (Hayes *et al.* 2006; reviewed in Rollins-Smith & Cohen 2005; Rollins-Smith 1998; Simmaco *et al.* 1997), and catecholamine (another stress hormone) exposure has been linked to accelerated growth of *A. hydrophila* in cultures (Kinney *et al.* 1999). But the growth inhibition of crowding can be ameliorated by ecological factors such as habitat complexity (Gromko *et al.* 1973; John & Fenster 1975; Rugh 1934) and schooling with kin (Jasieñski 1988; Pakkasmaa & Aikio 2003; Pakkasmaa & Laurila 2004; C.K.Smith 1990; D.C.Smith 1990). Kin association may reduce the impact of disease as kin selection theory predicts reduced competition among family members (Hamilton, 1964; Jasieñski, 1988), which would reduce the stress of high tadpole density. Conversely, kin association may increase the disease transmission within groups as any pathogen that infects one animal may be better able to infect its immunologically similar relatives (Hamilton 1987; Shykoff & Schmid-Hempel 1991).

The Role of the MHC in Disease Resistance and the Impact of Pathogens on Maintaining MHC Diversity

The MHC genotype of an individual and the level of MHC diversity in a population may have dramatic effects on the survival of individuals and of populations. The MHC is an extremely polymorphic gene complex primarily responsible for immunological self/non-self recognition, but is also involved in social behaviours such as mate

choice (reviewed in Bernatchez & Landry 2003; Piertney & Oliver 2006). Individuals with different MHC genotypes differ in their T-cell repertoires that recognise a different array of pathogens. In humans, specific MHC haplotypes have been linked to slowed HIV progression (Carrington *et al.* 1999) and reduced incidence of severe malaria (Hill *et al.* 1991). Similarly, certain MHC haplotypes are associated with slower progression of simian immunodeficiency virus (SIV) in macaques (Sauermann *et al.* 2000), and confer resistance or susceptibility to malaria in passerines (Bonneaud *et al.* 2006; Westerdahl *et al.* 2005). In Atlantic salmon, MHC haplotype determines resistance to a battery of diseases including infectious salmon anemia (Grimholt *et al.* 2003), infectious hematopoietic necrosis virus (Miller *et al.* 2004), and the bacterial pathogen *Aeromonas salmonicida* (Grimholt *et al.* 2003; Langefors *et al.* 2001; Lohm *et al.* 2002). In the only work of this nature in amphibians, Gantress *et al.* (2003) found specific MHC haplotypes that confer susceptibility and resistance of African clawed frogs (*Xenopus laevis*) to frog virus 3.

As MHC alleles are codominant (both alleles are expressed), individuals that are heterozygous at the MHC may have a greater immunological repertoire than do homozygotes (Doherty & Zinkernagel 1975). This heterozygote advantage may account for the high polymorphism found at the MHC. Heterozygote advantage has been documented in humans (Carrington *et al.* 1999), macaques (Sauermann *et al.* 2000), mice (McClelland *et al.* 2003; Penn *et al.* 2002) and Chinook salmon (Arkush *et al.* 2002). This fitness advantage may accumulate over an individual's lifetime. While certain MHC-homozygous genotypes may be more resistant to certain pathogens, MHC-heterozygous genotypes may cope better with sequential or simultaneous infections by different pathogens.

Rare allele advantage (negative frequency dependent selection, or the Red Queen hypothesis) is an alternative explanation for the high diversity of the MHC (Takahata & Nei 1990). According to the rare allele advantage hypothesis, there is considerable selection pressure on pathogens to avoid recognition by common host MHC haplotypes, which confers a selective advantage on individuals with rarer MHC haplotypes. Over time, the frequency of a common host haplotype will decline and be replaced by a once rare haplotype and selection will then drive pathogens to avoid recognition by the new common haplotype. There is model-based support for this hypothesis (Borghans *et al.* 2004; Satta *et al.* 1994; Takahata & Nei 1990). Detecting this process in empirical studies can be difficult because the selection pressure may be small and take many generations (Apanius *et al.* 1997; Satta *et al.* 1994).

Similarly, mating preferences can serve to maintain MHC polymorphism by individuals selecting mates with different MHC-genotypes to increase the number of MHC-heterozygous progeny (Penn *et al.* 2002; Penn 2002; Potts & Wakeland 1990), or by providing an intergenerational 'moving target' of MHC genotypes that would provide pathogen resistances to offspring that are distinct from both parents (Penn & Potts 1999). Alternatively, as the MHC is exceptionally polymorphic it may facilitate kin discrimination, MHC-disassortative mating and inbreeding avoidance (inbreeding avoidance hypothesis) (Brown & Eklund 1994).

Despite the importance of the MHC, very little is known about the role it plays in the disease resistance of amphibians. *Xenopus* and the axolotl (*Ambystoma mexicanum*) are the only amphibians in which the MHC has been characterized (Flajnik &

Kasahara 2001), and only one study has directly tested for MHC based resistances or susceptibility in amphibians (Gantress *et al.* 2003). Furthermore, no research in any host has experimentally exposed hosts to the natural microbial fauna associated with different MHC types, which can be locally adapted to that genotype. Depending on the stage of local adaptation, among other factors, locally adapted pathogens may be more virulent to local hosts, or populations of hosts may have adapted to local pathogens and be less susceptible to them than foreign host populations (Dybdahl & Storfer 2003).

In this thesis I explore the ecological and genetic factors that affect amphibians' ability to resist disease. All of these chapters are written for publication. In Chapter 2, I describe an experiment that examines the roles of crowding, habitat complexity and the kin composition of tadpole groups on the ability of these tadpoles to resist disease from the ubiquitous bacterial pathogen, *A. hydrophila.* In Chapter 3, I present the results of two experiments designed to test the importance of the MHC in disease resistance to *A. hydrophila*. In Chapter 4, I expand my examination of the MHC's role in disease resistance to include the importance of locally adapted microorganisms by exposing tadpoles to water preconditioned by adults of different MHC genotypes. In Chapter 5, I present an inventory of bacteria found in and on amphibians that have died in New Zealand and discuss their importance in terms of amphibian disease and probiotics.

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CHAPTER 2: THE ROLES OF KINSHIP, CROWDING, AND HABITAT COMPLEXITY ON THE GROWTH AND SURVIVAL OF *XENOPUS LAEVIS* TADPOLES

Abstract

Pathogens underlie most recent amphibian population declines, but little is known about the factors that predispose amphibians to infection. I examined the effects of crowding, kin composition (the relatedness of individuals in a group), and habitat complexity on the growth and survival of *Xenopus laevis* tadpoles exposed to the bacterial pathogen *Aeromonas hydrophila*. Tadpoles were reared in high or low density, with their siblings or with unrelated tadpoles, and in simple or complex habitats. Tadpoles then were exposed either to *A. hydrophila* or a sham inoculum. Tadpoles exposed to the bacterium were significantly smaller and more likely to die than tadpoles exposed to the sham. Tadpoles reared with siblings were larger, less variable in size, and had lower mortality rates than tadpoles reared in mixed sibship groups. The size difference between pure and mixed sibship groups was most pronounced when they were exposed to the pathogen. Habitat complexity reduced size variation within tanks but did not affect mean tadpole size. Mixed kinship composition and high tadpole density may increase competition, reduce growth, and increase disease susceptibility. My results indicate that growth is inhibited by pathogen exposure but that this effect may be ameliorated by reduced competition within groups of siblings.

Introduction

Amphibian populations have declined worldwide and infectious organisms are the most likely cause (Carey 2000; Daszak *et al.* 1999; Houlahan *et al.* 2001; Lips *et al.* 2006; Mendelson *et al.* 2006; Pounds *et al.* 2006; Stuart *et al.* 2004; Waldman & Tocher 1998). The bacterium *Aeromonas hydrophila* was long considered the causative agent in localized amphibian population die-offs (Bradford 1991; Frye 1985; Mauel *et al.* 2002; Nyman 1986; Russell 1898). More recently, iridoviruses (Cunningham *et al.* 1996; Green *et al.* 2002) and the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Berger *et al.* 1998; Burrowes *et al.* 2004; Lips *et al.* 2006; Mendelson *et al.* 2006; Pounds *et al.* 2006; Rachowicz *et al.* 2006; Smith *et al.* 2006; Stuart *et al.* 2004; Woodhams *et al.* 2006) have been hypothesised to be the primary disease agents in die-offs. If true, *A. hydrophila* may represent a secondary, opportunistic pathogen (Carey 2000; Cunningham *et al.* 1996; Hird *et al.* 1981). Why amphibians become susceptible to these pathogens is not clear. Antimicrobial peptides present in amphibian skin are effective at killing *B. dendrobatidis*, but not *A. hydrophila* (Rollins-Smith *et al*. 2002), and lethal bacterial infections usually occur in immunocompromised individuals (Taylor *et al*. 2001). Despite recent concern over global amphibian declines, the factors that predispose amphibians to opportunistic pathogens have received minimal attention.

Tadpole growth and development are affected by ecological factors (reviewed in Alford 1999), including crowding (Wilbur 1976, 1977; Wilbur & Collins 1973), habitat complexity or 'psychological space' (Gromko *et al.* 1973; John & Fenster 1975), and the kin composition of groups (Hokit & Blaustein 1997; Jasieñski 1988; Pakkasmaa &

Laurila 2004; Smith 1990b; Waldman 1991). Crowding increases competition and physiological stress (Glennemeir & Denver 2002), which inhibits immune system function (Hayes *et al.* 2006; Keller *et al.* 1981, 1983; Rollins-Smith & Cohen 2005; Rollins-Smith 1998). Conversely, kin association (reviewed in Jasieñski 1988; Pakkasmaa & Aikio 2003; Waldman 1991) and habitat complexity (Gromko *et al.* 1973; John & Fenster 1975; Rugh 1934) can reduce competition, physiological stress, and might help maintain healthy immune system function. However, kin association could be potentially detrimental. Kin association might increase the risk of disease transmission, as pathogens exploit shared immune system vulnerabilities of genetically similar hosts (Hamilton 1987; Shykoff & Schmid-Hempel 1991). In cannibalistic species such as *X. laevis* (Measey 1998; Tinsley *et al.* 1996), kin association also might lead to the cannibalism of relatives, and to the consequent ingestion of pathogens pre-adapted to a similar immune system (Pfennig *et al*. 1998).

The effects of kin association on fitness vary among species and ecological conditions. Tadpoles reared with kin can grow to be larger (Jasieñski 1988; Pakkasmaa & Aikio 2003; Pakkasmaa & Laurila 2004; C.K.Smith 1990; D.C.Smith 1990) or smaller (Hokit & Blaustein 1994; Shvarts & Pyastolova 1970a, 1970b) than tadpoles reared with non-kin, or may show no difference in size (Pakkasmaa & Laurila 2004; Travis 1980). Groups of tadpoles reared with kin may be more (Waldman 1991) or less variable in size (Jasieñski 1988; Pakkasmaa & Aikio 2003) than those reared in mixed groups. Variability within groups can indicate greater interference competition (Pakkasmaa & Aikio 2003; Weiner 1990) or nepotistic competitive self-restraint (Waldman 1991). I would expect that tadpoles in sibling groups that suffer less

competition, would be less stressed, less likely to suffer from immunosuppression, and thus more resistant to infectious disease.

Crowding is well known to inhibit tadpole growth (Alford 1999) although the mechanisms of growth inhibition may be diverse. Crowding may retard growth by causing stress among tadpoles due to their social interactions (John & Fenster 1975) including direct aggression (Smith 1990a; Waldman 1982), competition for limited resources (Bardsley & Beebee 2000; Petranka & Sih 1986; Travis 1984), or through secreted compounds or parasites (Richards 1958; Rose 1960; Steinwascher 1979a; Steinwascher 1979b). Crowding inhibits growth by increasing corticosterone levels (Glennemeir & Denver 2002). High corticosterone levels compromise both adaptive (Hayes *et al.* 2006; Rollins-Smith 1998) and innate immune function in amphibians (reviewed in Rollins-Smith & Cohen 2005; Simmaco *et al.* 1997). Furthermore, exposure to catecholamines – a class of hormones that includes adrenaline – increases the growth rate of the bacterial amphibian pathogen *A. hydrophila* (Kinney *et al*. 1999). The combined effects of compromised immune function and increased rate of bacterial growth due to the physiological responses to crowding might increase the susceptibility of stressed amphibians to infection by opportunistic pathogens such as *A. hydrophila* and possibly other pathogens, including *B. dendrobatidis* and iridoviruses.

Bacteria that are harmless under normal conditions can become pathogenic if animals are immunosuppressed. Pesticides, temperature, and crowding can compromise the immune system of amphibians (Carey & Bryant 1995; Hayes *et al.* 2006; Taylor *et al.* 2001). More generally, any chronic stressors, such as crowding, that increase

corticosterone or other corticoid levels can cause immunosuppression and increase disease susceptibility (Goulson & Cory 1995; Keller *et al.* 1981; Pai *et al.* 1995; Rigney *et al.* 1978; Rollins-Smith & Cohen 2005; Taylor *et al.* 2001; Waldman & Tocher 1998; Wedemeyer 1997).

With the dramatic worldwide decline of amphibians from infectious organisms it has become urgent to determine what factors are involved in moderating disease susceptibility. To do so, I examined whether crowding, habitat complexity, and the kin composition of groups affect the ability of *X. laevis* tadpoles to resist or effectively respond to infection by the bacterium *A. hydrophila*.

Materials & Methods

Subjects

Wild *Xenopus laevis* were collected in April 2003 from irrigation dams in Paarl, Stellenbosch, Somerset West, Caledon, and Wellington in the Western Cape Province of South Africa. For this experiment, I bred four pairs of frogs, twice, 1 week apart (broods A and B). On the day of breeding, between 13:00 and 15:00, I isolated and primed females by injecting 0.03 mg Luteinizing Hormone – Releasing Hormone (LH-RH; Argent Chemical Laboratories, Redmont, WA, USA) dissolved in 150 µL of sterilized water into their dorsal lymph sac. I monitored the cloacae of the frogs from 5 to 8 h after priming. Once cloacae displayed swelling and red colouration from increased blood flow, I injected the females with an additional 0.1 mg LH-RH dissolved in 500 µL of sterilized water and placed them into breeding tanks. I placed male frogs into the breeding tank with the female after they were injected with 0.03 mg LH-RH

dissolved in 150 µL of sterilized water. To ensure that the breeding pair would not consume the eggs, the substrate of breeding tanks was covered by a plastic mesh grid to allow fertilised eggs to fall through to the bottom. Adults were bred twice to ensure enough healthy tadpoles were available for the experiment. I used 600 tadpoles from each pair of adults, 400 from brood A and 200 from brood B. Tadpoles were kept in tanks (560 x 340 x 230 mm, high-density polyethylene) with a 12:12 h L:D photoperiod, and fed *ad libitum* with a filtered suspension of ground nettle. Aged, non-chlorinated, filtered municipal water, from underground aquifers, was used (19.8°C, ± 0.2°C).

After 2 weeks, I introduced tadpoles into rearing tanks with 5 L of water, in a 10:14 h L:D photoperiod. I stirred water in the source tanks to minimize selection bias (Pakkasmaa & Aikio 2003) and transferred tadpoles by net, taking care to avoid using any overtly unhealthy tadpoles. Tadpoles were placed into the following conditions (summarized in Table 2.1) in a fully crossed design: high or low social density (32 or 8 tadpoles per tank, 60 tanks each); physically partitioned (increased habitat complexity) or “bare” tanks (60 tanks each); with siblings (96 tanks, 24 tanks of each sibship) or in mixed-sibship groups (with equal numbers of the four sibling groups) (24 tanks); and exposure to pathogen or sham (60 tanks each). A total of 120 tanks with three replicates of every possible condition resulted.

Apparatus

Rearing tanks (340 x 290 x 180 mm, high-density polyethylene) were subdivided into small chambers by partitions (high habitat complexity) or not (bare). Partitions were constructed from three pieces of 4 mm thick opaque Perspex forming an extended ‘H’

shape and created six compartments (Fig. 2.1). The partitions' walls extended above water level, but did not touch the sides of the tank, which allowed tadpoles to move freely between compartments. Tadpoles acclimated to the new tanks for 2 days before the experiment began.

Bacteria

I isolated *Aeromonas hydrophila* from the heart of an adult *X. laevis* that died at the University of Canterbury. The isolate was identified by the New Zealand Ministry of Agriculture and Fisheries (MAF) Centre for Disease Investigation (Upper Hutt, New Zealand). The culture was grown in tryptone soya broth (TSB, Oxoid, Basingstoke, UK) aerobically for 24 hrs at 32°C in an incubator shaker. Bacterial density of cultures were quantified by triplicate serial dilution plate counts.

Procedure

I fed tadpoles daily for 23 days before exposing them to *A. hydrophila*. In weeks 1 and 2, I fed the tadpoles 50 mL of 0.5 Tbsp/L of ground nettle suspension per day. This was increased to 70 mL, 90 mL, 110 mL, 130 mL, and 150 mL in weeks 3 to 7 respectively. Feeding was *ad libitum*, as not all of the food was consumed before the next feeding (Denver *et al.* 1998; Merilä *et al.* 2004). I removed any tadpoles that died and replaced them with tadpoles of the same family, matched by size, to maintain constant social density.

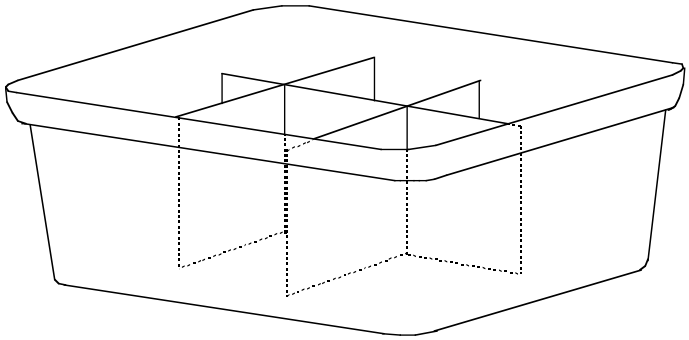

Figure 2.1 The partition used in the high habitat complexity condition.

Table 2.1 Breakdown of experimental conditions and the number of tanks in each condition in parentheses.

Sibship	Density	Habitat	Pathogen	Brood
Mixed Sibships (24)	High (12)	Simple (6)	No (3)	Early (2)
				Late (1)
			Yes (3)	Early (2)
				Late (1)
		Complex (6)	No (3)	Early (2)
				Late (1)
			Yes (3)	Early (2)
				Late (1)
	Low (12)	Simple (6)	No (3)	Early (2)
				Late (1)
			Yes (3)	Early (2)
				Late (1)
		Complex (6)	No (3)	Early (2)
				Late (1)
			Yes (3)	Early (2)
				Late (1)
Pure Sibships (96)	High (48)	Simple (24)	No (12)	Early (9)
				Late (3)
			Yes (12)	Early (9)
				Late (3)
		Complex (24)	No (12)	Early (9)
				Late (3)
			Yes (12)	Early (9)
				Late (3)
	Low (48)	Simple (24)	No (12)	Early (9)
				Late (3)
			Yes (12)	Early (9)
				Late (3)
		Complex (24)	No (12)	Early (9)
				Late (3)
			Yes (12)	Early (9)
				Late (3)

On day 23, I removed the tadpoles from their tanks and by net transferred them into a shallow, flat-bottomed porcelain tray (400 x 290 x 65 mm) with just enough water to cover the tadpoles, but not enough to allow them to swim at their normal 45-degree angle. I placed a ruler into the tray for calibration. I photographed the tray from directly above with a digital camera (Nikon Coolpix 4500, with 1024 x 768 pixel images, JPG format). I sterilized the tanks and partitions with 99% ethanol, allowed them to dry, refilled the tanks, and then returned the tadpoles to them. From then on, the tadpoles were exposed to bacteria laden food, or sham-infected food, as described below. On day 49, I photographed the surviving tadpoles again using the same methods.

Exposure

I exposed tadpoles on days 23, 33, 37, 41, and 45 to 10^6 colony-forming units (cfu)/ml, 2.5 x 10^6 cfu/ml, 5 x 10^6 cfu/ml, 7.5 x 10^6 cfu/ml, and 10^7 cfu/ml of *A. hydrophila*, respectively. Bacteria were centrifuged in 250 ml tubes for 15 min at 8000 rpm. The supernatant was removed and the pellet re-suspended in phosphate-buffered saline. This was repeated with un-inoculated TSB for the sham inoculum. The bacterial suspension, or sham suspension, was mixed with the daily food and added to the tank. Feeding and monitoring continued as normal. Tadpoles that died post-exposure were removed daily but were not replaced.

Measurements and Statistics

I measured tadpoles from digital images using ImageJ 1.3 (National Institutes of Health, Bethesda, Maryland; Abramoff *et al*. 2004). Body length (from the tip of the head to the base of the tail) and total length (from the tip of the head to the tip of the

tail) were measured for each tadpole. The mean length of all tadpoles in each tank was used as the experimental unit for analyses. To assess variation I calculated the adapted Levene's variable for each tank. The adapted Levene's variable is the mean absolute deviation from the tank median. The adapted Levene's variable offers a measure of variance robust to deviations from normality, and has superior Type I and II error properties to the coefficient of variation (Boos & Brownie 2004).

Length data were analyzed with five way repeated-measures GLMs with Type III sum of squares including all possible interactions (SAS 9.1.3, SAS Institute, Cary, North Carolina). Mortality data were analyzed with Poisson GLMs using the same model (R 1.5.1, R Foundation for Statistical Computing, Vienna, Austria). To test for difference in mortality among crowding conditions I used Gaussian GLMs on the arcsine square root transformed proportion of survival with Type III sum of squares including all possible interactions (SAS 9.1.3).

Results

Mortality

After exposure to *Aeromonas hydrophila*, more tadpoles died in mixed-sibship than in pure-sibship groups (Fig. 2.2a, $F_{1, 118} = 4.18$, $P = 0.041$), in bacteria-exposed than in sham-exposed treatments (Fig. 2.2b, $F_{1, 117} = 7.64$, $P = 0.0057$), and almost twice as many brood B than brood A tadpoles died (Fig. 2.2c, $F_{1, 115} = 111.07$, $P < 0.0001$). A greater proportion of tadpoles died in high-density than low-density treatments (Fig. 2.2d, $F_{1, 88} = 4.12$, $P = 0.045$). Higher mortality was associated with pathogen exposure in high-density than in low-density treatments (Fig. 2.3a. $F_{1, 88} = 4.84$, $P = 0.030$). Mortality did not differ significantly between partitioned and bare tanks.

Length

Overall, tadpoles reared with siblings were larger than those reared in mixed groups but this difference was not significant (see Table 2.2 for a summary of length data, body length [BL]: $F_{1, 88} = 2.92$, $P = 0.09$, total length [TL]: $F_{1, 88} = 2.81$, $P = 0.09$). Neither length measure differed significantly among the four pure sibship groups (BL: $F_{3, 32} = 2.52$, $P = 0.076$, TL: $F_{3, 32} = 2.523$, $P = 0.075$). Crowded tadpoles were about half the size of uncrowded tadpoles (BL: $F_{1, 88} = 1229.71$, $P < 0.0001$, TL: $F_{1, 88} = 1232.60$, $P < 0.0001$). Tadpoles exposed to *A. hydrophila* were significantly smaller than sham-exposed tadpoles (BL: $F_{1, 88} = 5.00$, $P = 0.028$). Brood B tadpoles were significantly smaller than brood A tadpoles (TL: $F_{1, 88} = 7.15$, $P = 0.0089$).

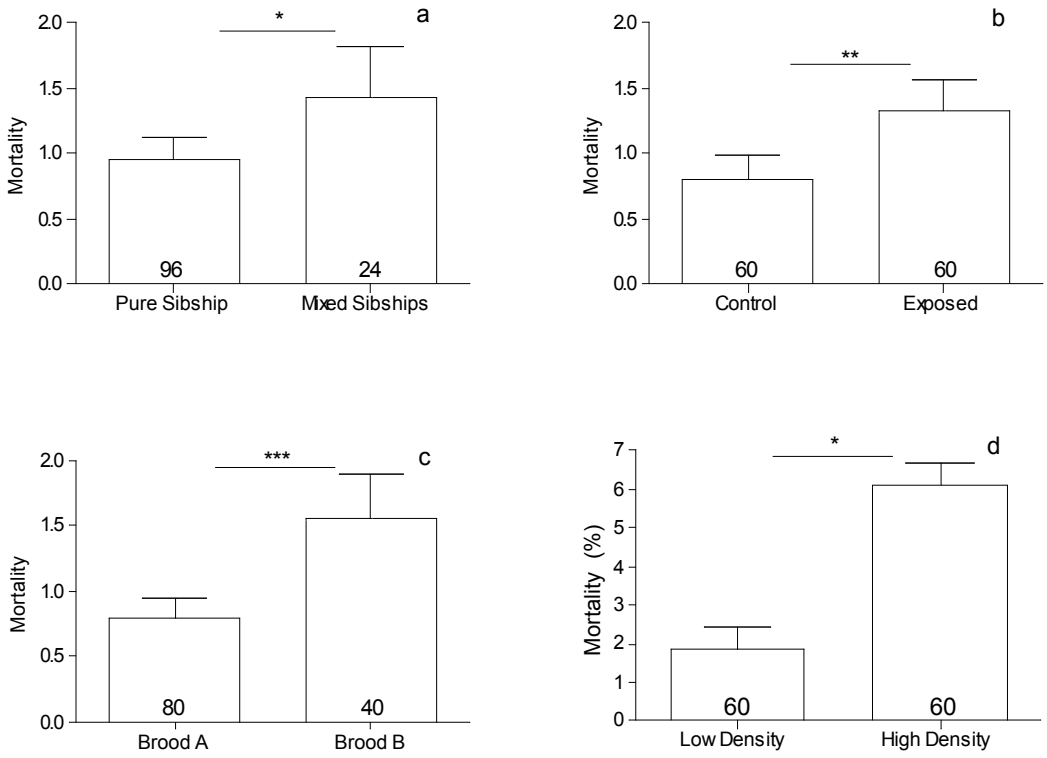

Figure 2.2 (a) Mean number of deaths per tank (± SE) in mixed and pure sibship groups after exposure to *Aeromonas hydrophila*, (b) mean number of deaths per tank (± SE) in groups exposed to *A. hydrophila* or the sham inoculum, (c) mean number of deaths per tank (± SE) in each brood after exposure to *A. hydrophila*, (d) mean percent mortality per tank (± SE) in low-density and high-density groups after exposure to *A. hydrophila*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Sample size is given at the base of each bar.

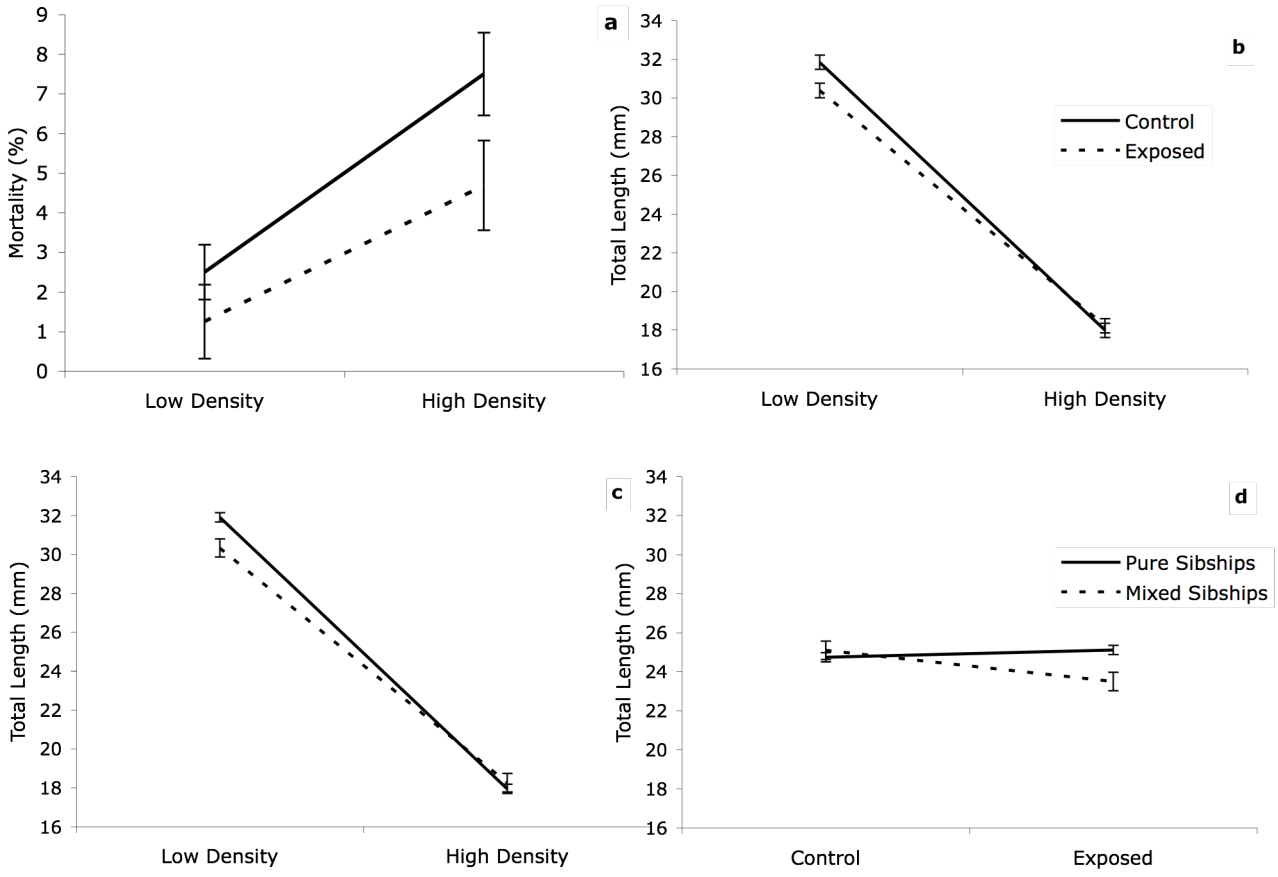

Figure 2.3 (a) Tadpole mortality per tank (±SE) among high and low density groups exposed to *A. hydrophila* or the sham inoculum, (b) mean tadpole total length (± SE) among high and low density groups exposed to *A. hydrophila* or the sham inoculum, (c) mean total length (± SE) of tadpoles in high or low density groups that were reared with their siblings or in mixed sibships, (d) mean total length (± SE) of tadpoles reared with their siblings or in mixed sibships and exposed to *A. hydrophila* or the sham inoculum.

The effect of pathogen exposure significantly differed in response to the level of crowding. Crowded tadpoles that were exposed to the pathogen did not differ in size from those exposed to the sham, but uncrowded tadpoles exposed to the pathogen

were smaller than the controls (Fig. 2.3b, BL: $F_{1, 88} = 5.71$, $P = 0.019$; TL: $F_{1, 88} = 5.22$, $P = 0.025$). Crowding also moderated the effect of kinship composition (Fig. 2.3c, BL: $F_{1, 88} = 7.69$, $P = 0.007$; TL: $F_{1, 88} = 6.52$, $P = 0.012$); crowded tadpoles reared with their siblings were not significantly different in size than those reared in mixed-sibship groups, but in low-density conditions, tadpoles reared with their siblings were larger than those reared in mixed-sibship groups. The kin composition of the tanks significantly affected the impact of disease exposure on growth (Fig. 2.3d, BL: $F_{1, 88} = 6.30$, $P = 0.014$; TL: $F_{1, 88} = 6.93$, $P = 0.010$). Pure-sibship groups exposed to a sham inoculum did not differ significantly in size from those exposed to the pathogen. In contrast, tadpoles exposed to the pathogen in the mixed-sibship conditions were smaller than the control tadpoles.

Variation

Kinship, crowding and habitat complexity also influenced the variation in size within tanks. Tadpoles reared in mixed-sibship groups were more variable in length than those reared in pure-sibship groups (Fig. 2.4a, BL: $F_{1, 88} = 21.52$, $P < 0.0001$, TL: $F_{1, 88} = 8.28$, $P = 0.005$). Mixed-sibship groups also had stronger positive skew than pure-sibships for all measures (BL day 23 skewness: mixed = 0.66, pure = 0.25; day 49: mixed = 0.12, pure = 0.07; TL day 23: mixed = 0.63, pure = 0.29; day 49: mixed = 0.06, pure = 0.05). Tadpoles reared at high density were less variable in size than those reared at low density (Fig. 2.4b, BL: $F_{1, 88} = 17.33$, $P < 0.0001$, TL: $F_{1, 88} = 15.79$, $P = 0.0002$). Tadpoles in partitioned tanks were less variable in size than those in bare tanks (Fig. 2.4c, BL: $F_{1, 88} = 5.57$, $P = 0.020$, TL: $F_{1, 88} = 6.67$, $P = 0.012$). However, variation in length was not significantly affected by exposure to the pathogen (BL: $F_{1,88} = 2.41$, $P = 0.12$, TL: $F_{1, 88} = 3.19$, $P = 0.078$) or brood (BL: $F_{1,88} = 1.10$, $P = 0.30$, TL: $F_{1, 88} = 0.007$, $P = 0.93$).

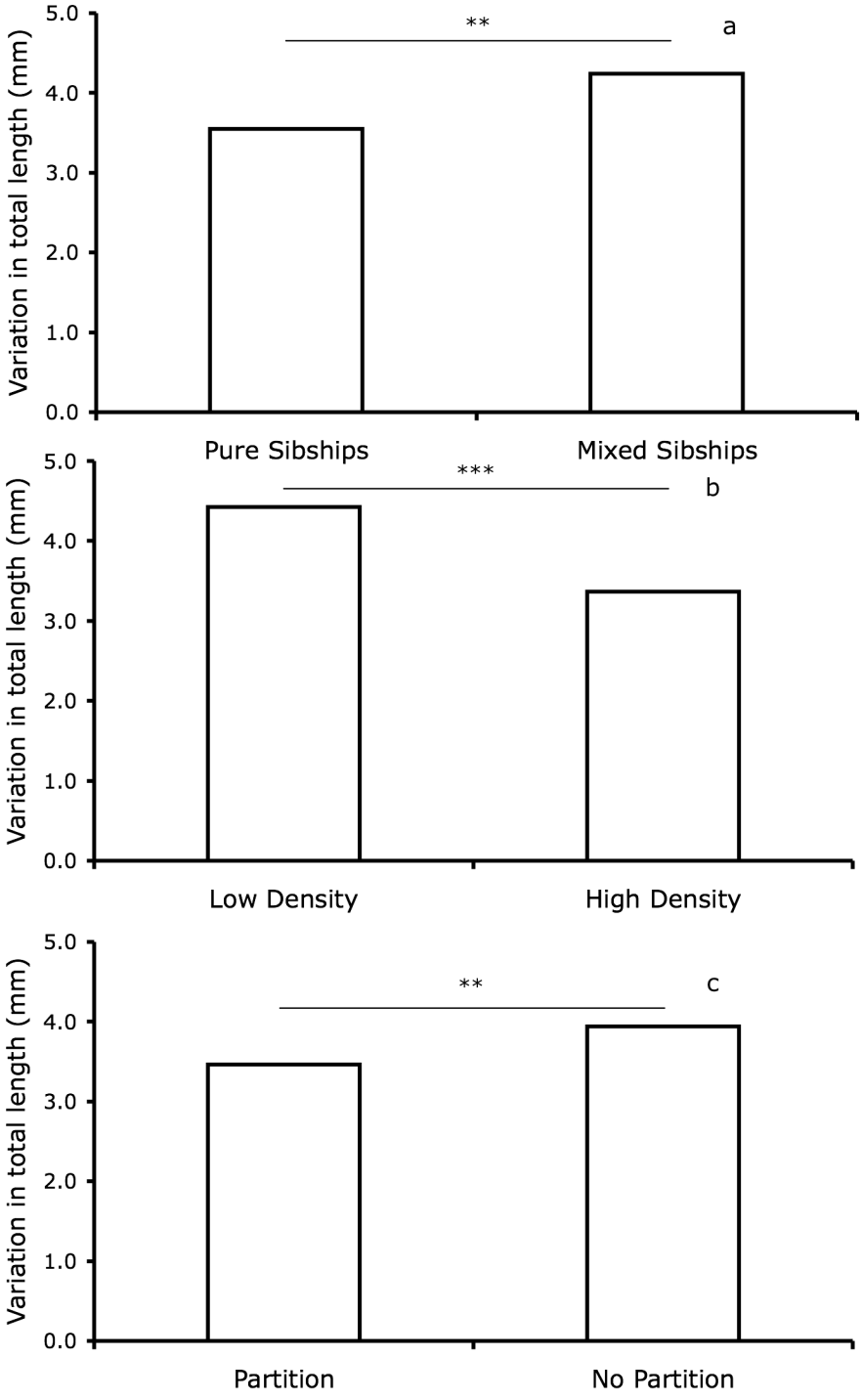

Figure 2.4 Mean absolute deviation from the median total length tadpoles in (a) **pure-sibship groups and mixed-sibship groups,** (b) **high and low-density groups,** (c) **and partitioned and bare tanks. *****P* **< 0.01, ******P* **< 0.001.**

Table 2.2 Body and total length before and after exposure to *A. hydrophila*. *F* statistics and *P* values are from repeated measures ANOVA.

Body Length	Measure One (Day 23)		Measure Two (Day 49)				
	Mean (mm)	SE	Mean (mm)	SE	df	*F*	*P*
Low Density	10.51	0.14	13.32	0.11	1, 88	1229.72	< 0.001
High Density	6.34	0.07	7.34	0.07			
Pure Sibships	8.47	0.23	10.34	0.32	1, 88	2.90	0.092
Mixed Sibships	8.24	0.46	10.27	0.58			
Bare	8.49	0.30	10.46	0.40	1, 88	0.067	NS
Partitioned	8.36	0.29	10.20	0.40			
Unexposed	NA	NA	10.40	0.41	1, 88	5.00	0.028
Exposed	NA	NA	10.26	0.40			
Early Brood	8.48	0.24	10.33	0.33	1, 88	2.80	0.098
Late Brood	8.32	0.38	10.33	0.53			

Total Length	Measure One (Day 23)		Measure Two (Day 49)				
	Mean (mm)	SE	Mean (mm)	SE	df	*F*	*P*
Low Density	27.78	0.38	35.38	0.31	1, 88	1231.60	< 0.001
High Density	16.92	0.16	19.53	0.21			
Pure Sibships	22.53	0.61	27.45	0.86	1, 88	2.90	0.092
Mixed Sibships	21.65	1.16	27.46	1.55			
Bare	22.56	0.76	28.16	1.10	1, 88	3.28	0.073
Partitioned	22.14	0.77	26.75	1.02			
Unexposed	NA	NA	27.36	1.07	1, 88	2.72	0.10
Exposed	NA	NA	27.55	1.06			
Early Brood	22.55	0.64	27.68	0.88	1, 88	7.15	0.0089
Late Brood	21.95	1.00	27.00	1.41			

Discussion

Crowding & Habitat Complexity

Tadpoles reared in crowded conditions were smaller than those in uncrowded conditions. Some crowded tadpoles were less than half the size of uncrowded ones. Moreover, a greater proportion of crowded than uncrowded tadpoles died, and this was most pronounced when the tadpoles were exposed to the pathogen, *Aeromonas*

hydrophila. Probably this is due to the elevated corticosterone levels associated with crowding and its effects on immune function.

John and Fenster (1975) suggested that the stress of crowding was due to increased interaction among tadpoles, and that increasing habitat complexity would reduce growth-retarding effects of crowding. My results are not consistent with this suggestion. Tadpoles reared in a more complex habitat did not show enhanced growth in either crowding condition, nor did they show increased survival after exposure to the pathogen. *Rana pipiens* tadpoles, separated by barriers, may have interacted less frequently (John and Fenster 1975) but *Xenopus* tadpoles are ecologically quite different. They are not known to display any form of agonistic behaviour toward conspecifics, and such interactions would appear unlikely because these tadpoles are obligate filter feeders and lack keratinised mouthparts. *Xenopus laevis* tadpoles probably do not respond to habitat complexity in the same manner as *R. pipiens* tadpoles. As *X. laevis* do not behave agonistically, the stress caused by perceiving conspecifics nearby would be lower than in a species known to bully conspecifics.

Even if tadpoles are not physically crowded, recent studies suggest that they can suffer the deleterious effects of crowding if their senses are “tricked” into thinking that they are crowded. *Xenopus laevis* and *Rana sylvatica* tadpoles presented with clay model tadpoles grew to a smaller size than control tadpoles without exposure to this stimulus (Rot-Nikcevic *et al.* 2005, in press). The *R. sylvatica* tadpoles exposed to the crowding stimuli had elevated levels of whole-body corticosterone which suggests that the stressful effects of crowding are possible even in the absence of true competition.

Although the *X. laevis* tadpoles in Rot-Nikcevic *et al.*'s study were not assayed for corticosterone it is likely that they would also demonstrate increased corticosterone levels with greater crowding stimuli. In turn, I would predict that they would be more prone to infectious disease.

Kinship

Tadpoles reared with their siblings grew more rapidly and were less likely to die than tadpoles reared in mixed-sibship groups after exposure to *A. hydrophila*. This benefit of kin association is likely to be context-dependent as at least two conflicting effects are potentially at work.

Tadpoles that associate with kin may treat their shoal-mates nepotistically and thus experience lower competitive intensity than those in mixed-sibship groups (Jasieñski 1988), which in turn may reduce the stress of crowding and the impact of disease. Conversely, the risk of disease transmission among kin may be higher, as pathogens that have successfully infected one individual can more easily infect its immunologically similar siblings than unrelated individuals (Hamilton, 1987; Pfennig *et al.* 1998; Shykoff & Schmid-Hempel 1991; Waldman & Tocher 1998).The influence of each of these effects may depend on the larval ecology of the species, the degree of host specificity, and the rate of pathogen local adaptation.

The interaction between crowding and kinship suggests that kin association is costly and that its benefits are context-dependent. Similar to Pakkasmaa & Aikio (2004), I found that siblings grow faster together under ideal, uncrowded conditions. Under crowded conditions, this benefit disappears. In high-quality environments, rapidly

growing tadpoles may restrict the intensity of their competition with siblings, thereby increasing their siblings' chances of survival at a small cost to their own growth. In crowded conditions, the cost of an individual's reduced feeding may exceed the benefit of nepotistic competitive self-restraint on inclusive fitness (Griffin *et al*. 2004). Despite the costs associated with kin association under suboptimal conditions, tadpoles reared with siblings suffered less growth inhibition than mixed-sibship groups when exposed to *A. hydrophila*, which suggests that some benefits of kin association may continue even when conditions deteriorate.

Brood Order

My results suggest that the fitness of *X. laevis* tadpoles from sequential broods may differ. Tadpoles from later broods were smaller and more likely to die after exposure to the bacterial pathogen. It is unclear what causes this difference. Eggs from early breedings may be higher quality than eggs from later breedings; for example, the later eggs may not be fully provisioned with, or have poorer quality, yolk. Tadpoles from large, well provisioned eggs often are conferred competitive advantages (Crump 1984; Dziminski & Alford 2005, 2006; Kaplan 1980; Parichy & Kaplan 1992; Tejedo & Reques 1992a). Mature *X. laevis* ovaries contain eggs in all stages of development (Smith *et al.* 1991). *Xenopus* breed several times in a season (Hey 1949), although it is unclear how common multiple breeding is in the wild or how much time separates these matings. Multiple breeding may be beneficial only under conditions that ensure the subsequent broods' survival, or as insurance against high mortality within the earlier brood. Given the lack of ecological data for *Xenopus* I cannot know whether two broods one week apart exceed normal breeding patterns, or if sequential broods have different fitness in the wild.

Variation and Interference Competition

Habitat complexity, although it did not affect mean tadpole growth, did appear to cause reduced size variation among individuals within groups. High variation in growth is associated with interference competition (Pakkasmaa & Aikio 2003; Weiner 1990). Although increasing habitat complexity failed to increase growth rates, the introduced barriers to both visual and tactile cues may have reduced interference competition among tadpoles.

Whether *X. laevis* tadpoles are subject to interference competition, especially given that they appear not to engage in any discernible social interactions, is unclear. Interference competition has been well documented among anuran larvae and often occurs indirectly through chemical secretions or commensal gut microfauna (but see Bardsley & Beebee 2000; Brown & Rosati 1998; Richards 1958; Steinwascher 1979a, 1979b) or through agonistic behaviours (Smith 1990a; Waldman 1982). As described above, agonistic behaviour is unlikely in *Xenopus* tadpoles and evidence for interference competition in *X. laevis* tadpoles is limited. Brown and Rosati (1998) interpreted reduced growth of tadpoles exposed to faeces of adult *X. laevis* as evidence of interference competition. However, introducing faeces into the tadpoles' water might have caused fouling or increased bacterial loads that would have the same effect on tadpole growth. Whether interference competition is mediated by the faeces of larval *X. laevis* remains unknown.

The findings of Rot-Nikcevic *et al.* (2005), discussed above, suggest that interference competition might occur in *X. laevis* tadpoles. Even in the absence of agonistic

behaviours or chemical interference, competition may come about due to purely psychological effects. Large tadpoles are more visually and tactilely conspicuous which may stress and inhibit smaller tadpoles' growth.

The greater variation within uncrowded and mixed-sibship groups, as compared to crowded and pure-sibship groups, indicates that interference competition may be greater in mixed-sibship groups and under low-density conditions (Pakkasmaa & Aikio 2003; Weiner 1990). Although some of the variation in mixed-sibship groups may be due to variation among families, I did not find any significant differences in size among the different pure-sibship groups. Interference competition can occur when resources are abundant (Birch 1957) and it is less likely to be the primary mode of competition when resources are limited, especially in this species, as competitors cannot be excluded from the diffuse food source. Despite my attempts to provide food in excess, food still may have been limiting which could be responsible for the reduced variation in high-density tanks.

Uniform growth in kin groups may be especially adaptive in *X. laevis,* as metamorphosed *X. laevis* are known to cannibalise conspecifics (Measey 1998; Parker *et al.* 1947; Tinsley *et al.* 1996). But young frogs are gape limited and often are smaller than large tadpoles. Tadpoles that grow up with their siblings would be closer in size and recently metamorphosed frogs would be unable to eat their tadpole siblings. The cannibalism of kin is especially costly. Not only does preying upon kin reduce the predator's inclusive fitness, but it may also increase the risk of contracting disease (Pfennig *et al.* 1998). However, if tadpoles grow up in mixed-kinship conditions, rapid development and metamorphosis could be advantageous. Early

metamorphosing frogs could utilize a new food resource while limiting their future competition (Crump 1983, 1990), and adults would be able to indirectly access larval food resources (Tinsley *et al.* 1996).

Conclusion

Despite the considerable concern about global amphibian declines, few studies have examined the ecological factors that contribute to amphibian disease. Crowding and shoal kin composition (described here), pesticide exposure (Carey & Bryant 1995; Christin *et al.* 2004; Gendron *et al.* 2003), population level genetic diversity (Hedrick 2002; Hedrick *et al.* 2001), and inbreeding (Acevedo-Whitehouse *et al.* 2003; Coltman *et al.* 1999; Potts *et al.* 1997) are all linked to disease susceptibility in many taxa, and are likely to interact in a complex fashion. The synergistic effects of various ecological conditions cannot be underestimated and further research needs to address these (Waldman & Tocher 1998). I have shown that kin composition, habitat complexity, crowding, and breeding order affect tadpole growth and disease susceptibility. These and other ecological and genetic factors can predispose amphibian populations to declines and extinction from infectious agents.

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CHAPTER 3: MHC BASED SUSCEPTIBILITY TO A BACTERIAL PATHOGEN IN AFRICAN CLAWED FROG (*XENOPUS LAEVIS*) TADPOLES

Abstract

Given recent cataclysmic amphibian declines from infectious disease, understanding the mechanisms involved in amphibian disease resistance is becoming increasingly urgent. The major histocompatibility complex (MHC) is an integral part of the vertebrate adaptive immune system and has been well characterized in *Xenopus laevis*. To elucidate the importance of the MHC in conferring disease resistance, I challenged *X. laevis* tadpoles with different combinations of four MHC haplotypes (*f*, *g*, *j*, and *r*) to the bacterial pathogen, *Aeromonas hydrophila*. I found that exposure to *A. hydrophila* reduced the survival of these tadpoles and that individuals with *r* or *g* MHC haplotypes were more susceptible than tadpoles with *f* or *j* haplotypes. Tadpoles with different MHC genotypes also grew at different rates. Tadpoles that were heterozygous at the MHC with both susceptible and resistant haplotypes were always intermediate to either homozygotes in both size and survival. These results demonstrate that this bacterium affects the growth and survival of *X. laevis* tadpoles, and that the MHC moderates these effects.

Introduction

The major histocompatibility complex (MHC) encodes cellular mechanisms that facilitate immunological self/non-self recognition. Individuals with different MHC genotypes differ in their T-cell repertoires, which recognise different arrays of pathogens. As MHC alleles are codominant, individuals that are heterozygous at the MHC may have a greater immunological repertoire than do homozygotes (Doherty & Zinkernagel 1975). This fitness advantage may accumulate over an individual's lifetime. While particular MHC-homozygous genotypes may be more resistant to certain pathogens, MHC-heterozygous genotypes may cope better with sequential or simultaneous infections by different pathogens (McClelland *et al.* 2003; Penn *et al.* 2002).

The MHC of amphibians is poorly understood, with two exceptions: the South African clawed frog *Xenopus laevis*, and the axolotl *Ambystoma mexicanum* (Flajnik & Kasahara 2001). *Xenopus* MHC class I and II loci are closely linked (Liu *et al.* 2002; Nonaka *et al.* 1997). *Xenopus* tadpoles express MHC class I molecules only in the epithelial tissue of some organs such as gills, lungs, and intestine (Salter-Cid *et al.* 1998), and class II molecules on B cells and antigen-presenting cells (Flajnik *et al.* 1987). Despite their limited MHC class I expression, tadpoles are immunocompetent, although they are more susceptible than adults to viral infections (Gantress *et al.* 2003). Class I molecules present protein fragments from intracellular pathogens such as viruses (Bernatchez & Landry 2003; Salter-Cid *et al.* 1998), so the vulnerability of tadpoles to viruses may be associated with their limited class I expression.

The role of the MHC in amphibian disease resistance is particularly important now, given the dramatic worldwide decline of amphibians (Carey 1997, 2000; Daszak *et al.* 1999, 2003; Houlahan *et al.* 2001, 2000; Lips *et al.* 2006, 2004; Mendelson *et al.* 2006; Pounds *et al.* 2006; Stuart *et al.* 2004). Three pathogens have been identified as causal agents in die-off events: the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) (Berger *et al.* 1998; Bosch *et al.* 2001; Lips *et al.* 2006, 2004; Mendelson *et al.* 2006; Rachowicz *et al.* 2006), iridoviruses (Cunningham *et al.* 1996; Green *et al.* 2002), and the bacterium *Aeromonas hydrophila* (Bradford 1991; Frye 1985; Mauel *et al.* 2002; Nyman 1986; Russell 1898). However, *A. hydrophila* is currently considered a secondary pathogen (Carey 2000; Cunningham *et al.* 1996; Hird *et al.* 1981) that nonetheless is likely to infect immunocompromised animals (Taylor *et al.* 1999a, 2001).

Despite the importance of the MHC to vertebrate disease resistance and conservation, few studies have directly tested its role in amphibian disease (i.e. Gantress *et al.* 2003). I sought to examine whether MHC genotype affected the survival and growth of *X. laevis* tadpoles challenged with *A. hydrophila*. To do so, I exposed tadpoles that bore diploid combinations of four different MHC haplotypes with *A. hydrophila*. I measured the *A. hydrophila* resistance of different MHC genotypes across several families, compared the resistances of different MHC genotypes within families, and evaluated whether the particular haplotypes conferred different resistances.

Materials & Methods

Bacteria

I used a strain of *Aeromonas hydrophila* that I isolated from the heart of an adult *Xenopus laevis* that died at the University of Canterbury amphibian facility (Chapter 5). I cultured these bacteria on tryptone soya agar (TSA, Oxoid, Basingstoke, UK), and incubated the plates aerobically for 24 ± 4 h at 32°C. I introduced a single colony into a universal bottle of tryptone soya broth (TSB, Oxoid, Basingstoke, UK), and incubated this for 24hrs ± 4 h aerobically at 32°C. After incubation, I transferred 10 mL of the broth culture into 1 L of TSB in Erlin-Meyer flasks. I incubated these flasks aerobically for 24 ± 4 h at 32°C in a controlled environment incubator shaker shaking at 200 rpm. I quantified the cultures by triplicate serial dilution plate counts the day before experimental exposure.

Among Families Methods

Subjects & Procedure

I bred six MHC homozygous (*ff, gg, jj*) *X. laevis* frogs four times during one night. This produced 12 clutches of tadpoles with 6 genotypes (*ff, fg, fj, gg, gj, jj*). I used MHC heterozygous tadpoles that were half-siblings of the MHC homozygous tadpoles (i.e. *fg* tadpoles are half-siblings of *ff* and *gg* tadpoles) from inbred strains to limit non-MHC heritable differences. On the day of breeding, between 13:00 and 15:00, I isolated and primed females by injecting them into their dorsal lymph sac with 0.03 mg Luteinizing Hormone – Releasing Hormone (LH-RH; Argent Chemical Laboratories, Redmont, WA, USA) dissolved in 150 µL of sterilized water. I monitored the cloacae of the frogs from 5 to 8 h after priming. Once cloacae displayed swelling

and red colouration from increased blood flow, I injected the females with an additional 0.1 mg LH-RH dissolved in 500 µL of sterilized water and placed them into breeding tanks. To ensure that the breeding pair would not consume the eggs, the substrate of breeding tanks was covered by a plastic mesh grid to allow fertilised eggs to fall through to the bottom.

The laboratory strains I used were developed by Louis Du Pasquier and Martin Flajnik. These frogs have known sequences for MHC class I and II alleles within haplotypes defined as *f*, *g*, *j*, and *r*. The sequences for the MHC alleles are published on GenBank (accession numbers: AF185579, -80, -82, -86)(Flajnik *et al.* 1999).

I placed male frogs into the breeding tank with the female after they were injected with 0.03 mg LH-RH dissolved in 150 µL of sterilized water. I paired MHC-identical homozygotes first, and then after they had begun spawning, I separated the amplexed adults and allowed them to continue mating with partners with which they differed in MHC genotype. I subsequently repeated this protocol to control for egg order effects by creating early and late broods of each MHC genotype. Two days after hatching I placed 100 tadpoles from each brood into 10 L high-density polyethylene tanks.

Two weeks after hatching I photographed 384 tadpoles, 32 tadpoles from each clutch. Each tadpole was photographed in its own Petri dish from directly above (60cm) with a Nikon Coolpix 4500 digital camera. I measured body length (BL, from the tip of the snout to the vent at the base of the tail) and total length (TL, from the tip of the head to the tip of the tail) from photographs using NIH ImageJ 1.3 (National

Institutes of Health, Bethesda MD, USA). I subsequently placed each tadpole into an individual 1 L polypropylene beaker (day 0). I exposed tadpoles to an innoculum of *Aeromonas hydrophila* or a control. Experimental tadpoles were inoculated with either 1.0×10^6 colony forming units/ml (cfu/ml), 2.5×10^6 cfu/ml, or 3×10^6 cfu/ml of *A. hydrophila*. These doses do not exceed the density of this bacterium found in the wild (Hazen *et al*. 1978). Control tadpoles were inoculated with 3×10^6 cfu/ml of *A. hydrophila* that had been killed by autoclave at 121° C, 103 kPa for 20 min. Each treatment comprised 8 tadpoles from each clutch. Within two-replicate blocks (48 beakers in a 12 x 4 grid), I moved each beaker one place every day to prevent position effects. Tadpoles were fed every second day with an increasing amount of ground nettle suspension and the water was topped up to 1 L every 4 days to account for evaporation.

Ten, 25, and 35 days after exposure to the experimental conditions I photographed and measured the tadpoles as before. I analysed body and total length with repeated-measures univariate ANOVA with Type III sum of squares. MHC, bacterial dosage, oviposition time, and block were main effects and interactions among these effects were also analysed. I used orthogonal contrasts to compare the length of control tadpoles to tadpoles exposed to the bacteria. For within-individual analyses I used the Huynh-Feldt adjusted *P* value when the assumption of sphericity was violated. I analysed the mortality data with a fully factorial binomial generalized linear model (GLZ) with the same fixed factors using a Logit Link function to create maximum-likelihood ratio estimates. All analyses were conducted with Statistica 6.1 (Statsoft, Tulsa, OK, USA).

Despite using half-siblings from inbred lines of *X. laevis* to limit the heritable effects of non-MHC genes, these genes may still have had effects on the disease resistance of the tadpoles. To address this I conducted within family tests to control for non-MHC variation.

Within Families Methods

Subjects & Procedure

I crossed three pairs of MHC-identical heterozygote frogs (*fg* x *fg*, *fr* x *fr*, *rg* x *rg*) to produce broods consisting of mixed homozygotes and heterozygotes (e.g. *rr*, *rg*, *gg*). The following day I removed 200 eggs from each clutch and placed them individually into 1 L polypropylene beakers.

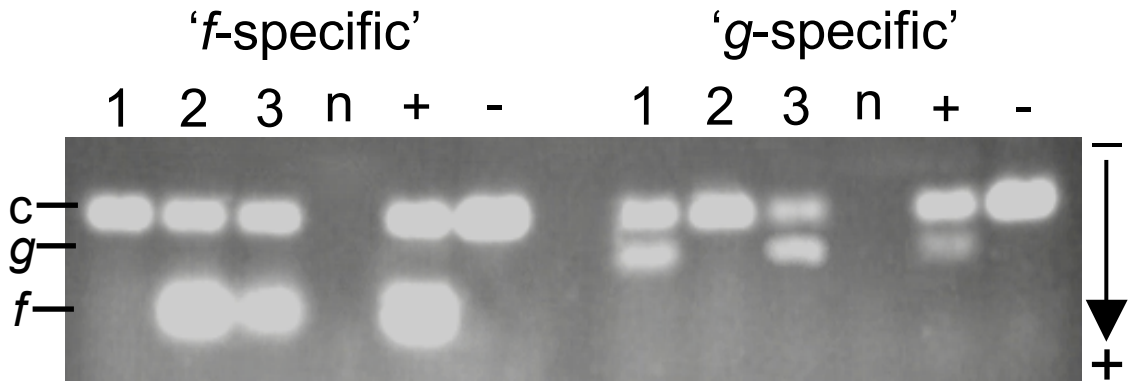
Genotyping

Two weeks after hatching I genotyped 150 tadpoles of each brood for MHC genotype. I cut a small section of the tail from 150 tadpoles of each clutch. As the tail quickly regenerates, the harm to the tadpoles appeared to be minimal. I extracted the DNA from the tail samples using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) by following the manufacturer's instructions and purified by salt precipitation. I then ran polymerase chain reaction (PCR) on the samples using MHC class I-α1 domain sequence-specific primers. As the class I and class II genes cosegregate (Liu *et al.* 2002) my methods indirectly genotype the animals at the class II locus. I did not know the MHC genotypes of subjects until after the experiment and analyses were complete.

I MHC-typed individuals by touchdown PCR using sequence-specific primers (SSP) that anneal to polymorphic sequences within the MHC class I-α1 domain (coding for the peptide-binding region, PBR) for each of the known alleles. In each SSP-PCR reaction, I included primers that amplify DNA from a conserved region of the MHC (class I-α3 domain) to control for any failed PCR reactions that would otherwise be falsely scored as negative (Bunce *et al.* 1995; Bunce & Welsh 1994; Gilchrist *et al.* 1998; Krausa *et al.* 1993). These control primers produce PCR product in all successful PCR reactions such that false negatives that may occur in PCR can be identified (Fig. 3.1). I designed primers using Primer3 (Rozen & Skaletsky 2000) from known *X.* laevis sequences (Flajnik *et al.* 1999) The *f*-haplotype-specific primers (for: GTC TCA GAT CGA GCC TTT GG, rev: TTG CAG GTT CAT CTC TAC CAG T) amplify a 106 base pair fragment. The *g*-haplotype-specific primers (for: GTC TCA GAT CGA GCC TTT GG, rev: GCT CTG ATC CCT TGG CAA T) amplify a 178 base pair fragment. The *r*-haplotype-specific primers (for: AGA TAG AGC ATT TGG GCT GC, rev: ATT CAG GTC CTG CTT TGT CC) amplify a 134 base pair fragment. The control primers (for: TCA CCC TCA TGT AAG AAT TTC AGA, rev: GCT CCA CAT GAC AGG CAT AA) amplify a 236 base pair fragment.

Sequences were amplified on 96-well PCR plates (Axygen Scientific, PCR-96-C) in 12.5 µL PCR reactions, each containing 50 ng of template DNA, PCR reaction buffer (63.6 mM KCl, 127.2 mM Tris-HCl (pH 8.3), 1.9 mM $MgCl_2$), 25 nmol $MgCl_2$, 180µM dNTP (100mM, Eppendorf, Hamburg, Germany) and 0.2 units Taq polymerase (Roche Diagnostics, Basel, Switzerland). Primer concentrations varied and depend on the haplotype being tested for. Each *f*-specific PCR contained 16.5 pmol of each *f*-specific primer and 3.5 pmol of each control primer. Each *g*-specific PCR contained

12.5 pmol of the *f*-forward primer, 20 pmol of the *g*-reverse primer, and 1 pmol of each control primer. Each *j*-specific PCR contained 15 pmol of the *j*-forward primer, 30 pmol of the *j*-reverse primer, and 0.75 pmol of each control primer. Each *r*-specific



PCR contained 21.25 pmol of each *r*-specific primer and 2.5 pmol of each control primer.

Figure 3.1. Sample of electrophoresed MHC-allele PCR products from three tadpoles of an *fg* x *fg* parental cross. All samples were tested for both the *f* and *g* MHC class I-α1 domain sequences in two sets of PCR's including control primers (c) from a conserved region of the MHC-class I-α3. Samples were electrophoresed with negative controls (n), known allele positives (+) and negatives (-). Sample '1' is a *gg* homozygote as only the g-allele sequence amplified. Sample '2' is an *ff* homozygote and sample '3' is an *fg* heterozygote.

The conditions for touchdown PCR in an Eppendorf Mastercycler gradient, thermocycler were as follows: denaturation for 90 s at 94˚C, followed by 5 cycles of denaturation for 30 s at 94˚C, annealing for 45 s at 70˚C and primer extension for 30

s at 72˚C, followed by 20 cycles of denaturation for 30 s at 94˚C, annealing for 50 s at 65˚C and primer extension for 45 s at 72˚C, followed by 5 cycles of denaturation for 30 s at 94˚C, annealing for 1 min at 56˚C and primer extension for 2 min at 72˚C.

I electrophoresed PCR products next to known positives and negatives for 40 min at 70 volts in horizontal 2% agarose gels. Gels were visualized by ethidium bromide fluorescence.

Procedure

Three weeks after hatching I photographed and measured the tadpoles as in the previous experiment and subsequently exposed them to *A. hydrophila*, heat killed *A. hydrophila*, or pelleted clean bacterial media. The pelleted clean bacterial media served as a second control that allowed me to assess whether dead bacterial inoculum, as used in the previous experiment, was an appropriate control. I exposed the tadpoles to an initial bacterial dose of 2×10^7 cfu/ml, the same amount of heat-killed bacteria, or a pellet from the same amount of clean media.

The numbers of each genotype that each family produced limited the sample size (Table 3.1). I arranged the beakers in single-family blocks five beakers across. Tadpoles in each row of beakers were the same genotype and in the same exposure condition; I moved each row one position every day within the family blocks to ensure that all tadpoles were exposed to the same position effects (e.g., differences in light and thus algal or other microbial growth).

Table 3.1 Within-families sample sizes of each genotype and exposure condition

Cross	Genotype	Exposed	Control (dead bacteria)	Control (clean media)
fg x *fg*	*ff*	10	10	-
	fg	10	10	11
	gg	10	10	-
fr x *fr*	*ff*	14	14	-
	fr	16	16	16
	rr	16	16	-
rg x *rg*	*rr*	15	15	-
	rg	16	16	16
	gg	13	13	-

On day 5 I exposed the tadpoles to 4 x 10^7 cfu/ml of *A. hydrophila*. On day 18 I photographed and measured all tadpoles and cut a small section of tail to create a portal for the bacteria, I re-photographed the tadpoles, and exposed them to 6 x 10^7 cfu/ml of *A. hydrophila*. On day 28 the experiment ended and I photographed and measured the tadpoles for the last time.

I compared the lengths of tadpoles in the two control groups separately with repeated-measures univariate ANOVA, I used the controls exposed to the dead bacteria as the controls in subsequent analyses. I analysed length data by repeated-measures univariate ANOVA with MHC genotype nested within family and exposure as fully factorial main effects, using type III sum of squares. For the within individual analyses I used Huynh-Feldt adjusted *P* values when the assumption of sphericity was violated. I analysed the mortality data with a fully factorial binomial generalized linear model (GLZ) with the same fixed factors using a Logit Link function to create maximum-likelihood ratio estimates. Finally I compared the length of tadpoles at day

0 from both experiments using a Student's t test assuming unequal variance (Statistica 6.1).

Results

Among Families

Length

Across all measurements, tadpoles with different MHC genotypes were significantly different in length (Fig. 3.2)(BL: $F_{5, 258} = 6.50$, $P < 0.001$; TL: $F_{5, 258} = 8.26$, $P < 0.001$). The largest and smallest tadpoles were of *gg* and *fj* MHC genotypes, respectively. This did not change as the experiment progressed. Tadpoles that developed from eggs oviposited earlier in the evening were significantly smaller than those oviposited later in the same evening (BL: $F_{1, 258} = 4.60$, $P = 0.033$, TL: $F_{1, 258} = 7.10$, $P = 0.008$). On day 25 tadpoles from the later broods were 4% larger than tadpoles from earlier broods (TL: early 21.07 mm ± 0.28 mm, late 21.92 mm ± 0.25 mm, mean ± SE), but by day 34 there was no difference in size between the two broods (BL: $F_{1, 258} = 0.43$, $P = 0.51$, TL: $F_{1, 258} = 0.59$, $P = 0.44$).

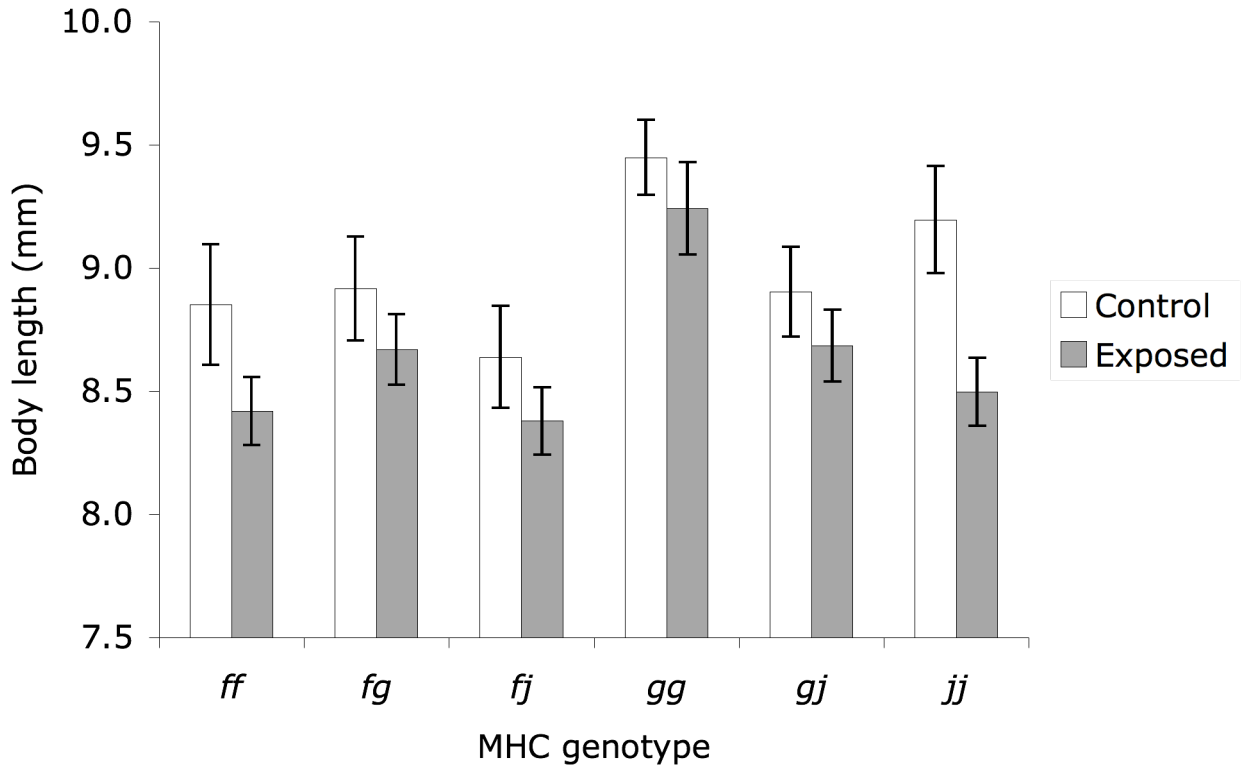


Figure 3.2 Body length (±SE) at day 25 of tadpoles from each genotype exposed to the pathogen *A. hydrophila* and the control.

Exposure dose of *A. hydrophila* did not significantly affect tadpole growth (BL: $F_{3, 258}$ = 2.42, P = 0.067, TL: $F_{3, 258}$ = 1.98, P = 0.12) although controls were significantly larger (TL: 22.36 ± 0.34 mm) than all those exposed (21.19 ± 0.22 mm) at day 25 (orthogonal contrast BL: $F_{1, 258}$ = 15.42, P = 0.0001; TL: $F_{1, 258}$ = 12.75, P = 0.0043). Thirty-four days after exposure, surviving tadpoles that were exposed to the pathogen were of similar size to the control tadpoles (orthogonal contrast BL: $F_{1, 258}$ = 0.0051, P = 0.94; TL: $F_{1, 258}$ = 0.108, P = 0.74).

Mortality

Tadpole mortality was significantly affected by exposure to *A. hydrophila*, the tadpoles' MHC genotype, and brood order. Tadpoles exposed to higher inoculums of *A. hydrophila* suffered higher mortality (Fig. 3.3)(χ^2 = 14.0, 3 d.f., P = 0.003). However, some MHC genotypes suffered less mortality than others (Fig. 3.4)(χ^2 = 18.25, 5 d.f., P = 0.0026). Furthermore, the influence of exposure dose on mortality differed with MHC genotype (χ^2 = 26.98, 15 d.f., P = 0.029). Certain MHC genotypes appear especially prone to *A. hydrophila; gg* tadpoles had the highest rate of mortality when exposed to the bacterium (43%) but none of the control tadpoles died. In contrast, *ff* tadpoles did not suffer increased mortality when exposed to the pathogen. The *fg* tadpoles had intermediate mortality rates to their *ff* and *gg* half-siblings (Fig. 3.4). A similar pattern is apparent in the *gg*, *gj*, and *jj* tadpoles, but not in the *ff*, *fj*, and *jj* tadpoles. Tadpoles that were from earlier clutches had significantly higher mortality than full siblings from later clutches (Fig. 3.5)(χ^2 = 10.52, 1 d.f., P = 0.0012).

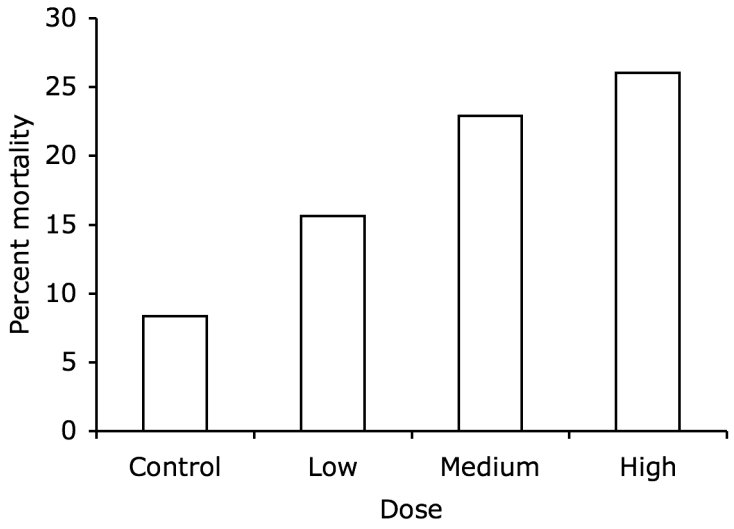


Figure 3.3 Percent mortality of tadpoles exposed to the control = 3 x 10^6 cfu/ml heat killed, low = 1 x 10^6 cfu/ml, medium = 2.5 x 10^6 cfu/ml, and high = 3 x 10^6 cfu/ml doses of *A. hydrophila*

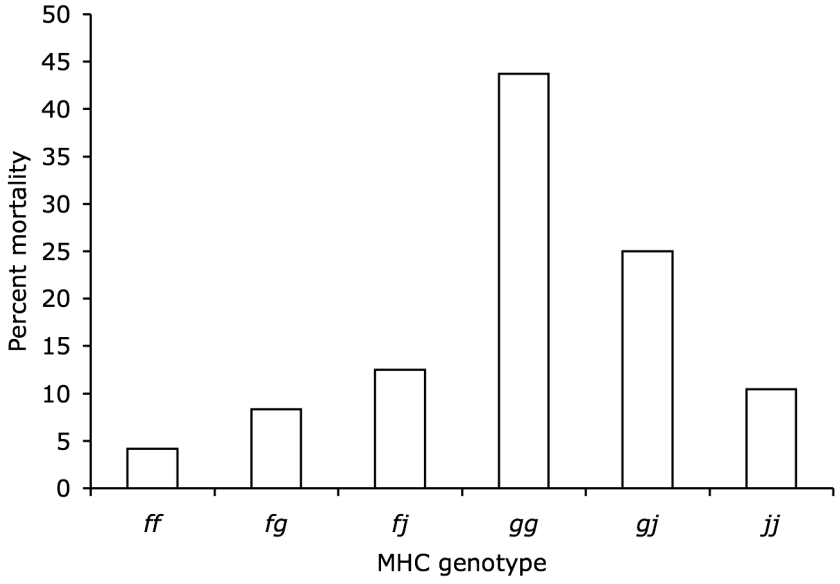


Figure 3.4 Percent mortality of tadpoles from each MHC genotype that were exposed to live *A. hydrophila*.

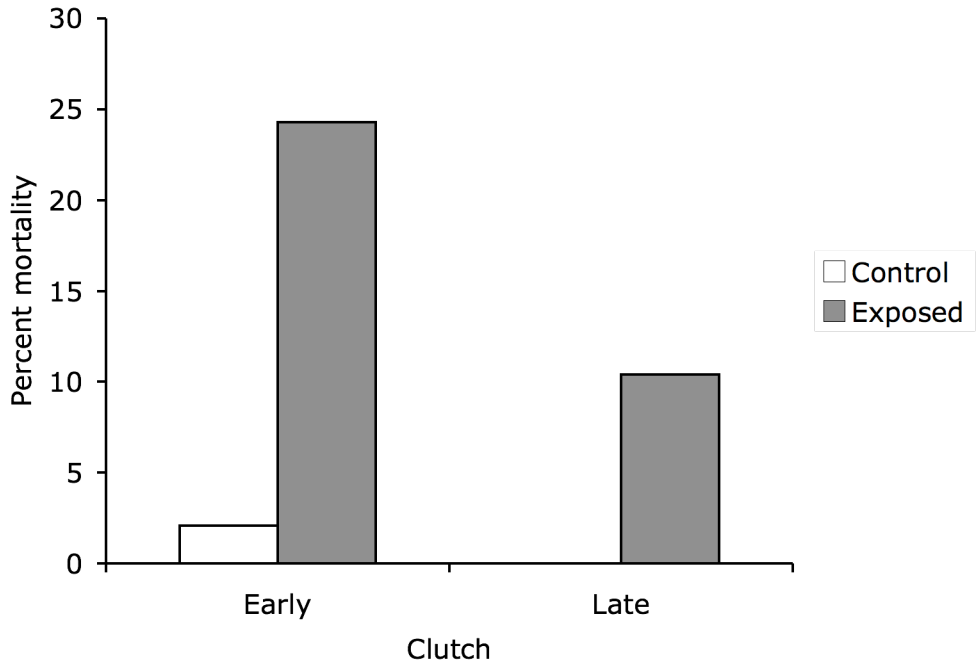


Figure 3.5 Percent mortality of tadpoles bred early and late in an evening.

Within Families

Length

MHC genotype did not significantly affect tadpole size in this experiment although it did affect the impact of pathogen exposure (Tables 3.2, 3.3). Tadpoles exposed to dead *A. hydrophila* were significantly larger than tadpoles exposed to pelleted clean media (Table 3.4, Fig. 3.6). Surviving tadpoles that had been exposed to live *A. hydrophila* also were significantly larger than tadpoles exposed to the dead bacteria (Fig 3.5). Tadpoles with different MHC genotypes did, however, responded differently to the bacterial challenge (Table 3.2, Fig. 3.7)(day 18: BL, $F_{8, 159} = 2.18$, $P = 0.032$; TL, $F_{8, 159} = 2.28$, $P = 0.025$). Each individual's growth across the experiment was

affected by their exposure condition and their MHC genotype (Table 3.4). There were also significant differences among growth rates of MHC genotypes dependent on their exposure condition (Fig. 3.8a-c). Tadpoles with different MHC genotypes grew at different rates when exposed to this pathogen but no clear trend was apparent. Tadpoles in the within-families experiment were significantly larger (BL 6.02 ± 0.06 mm) than tadpoles in the among-families experiment (BL 5.22 ± 0.03 mm; t = 12.63, 626 d.f., $P < 0.0001$).

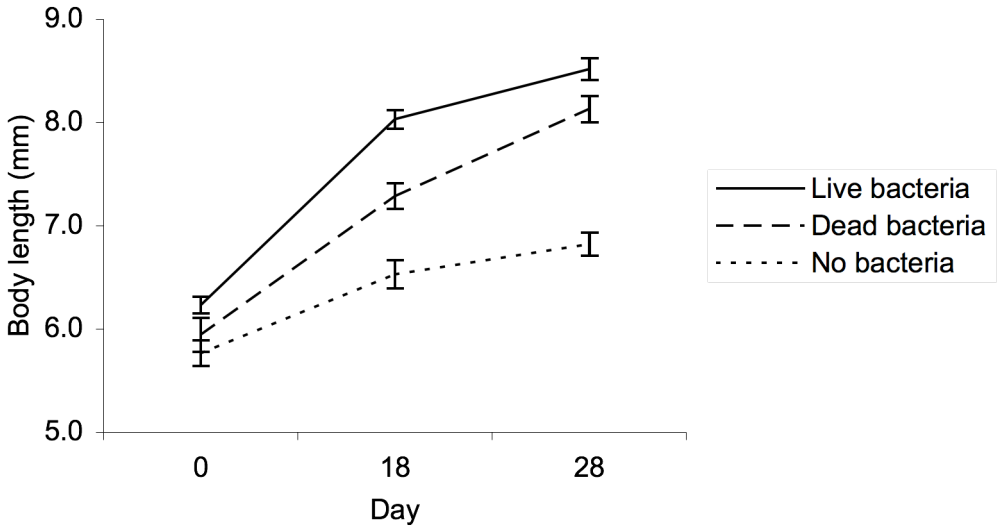


Figure 3.6 Body length (± SE) of tadpoles exposed to live *Aeromonas hydrophila*, and the two controls: dead bacteria, and no bacteria.

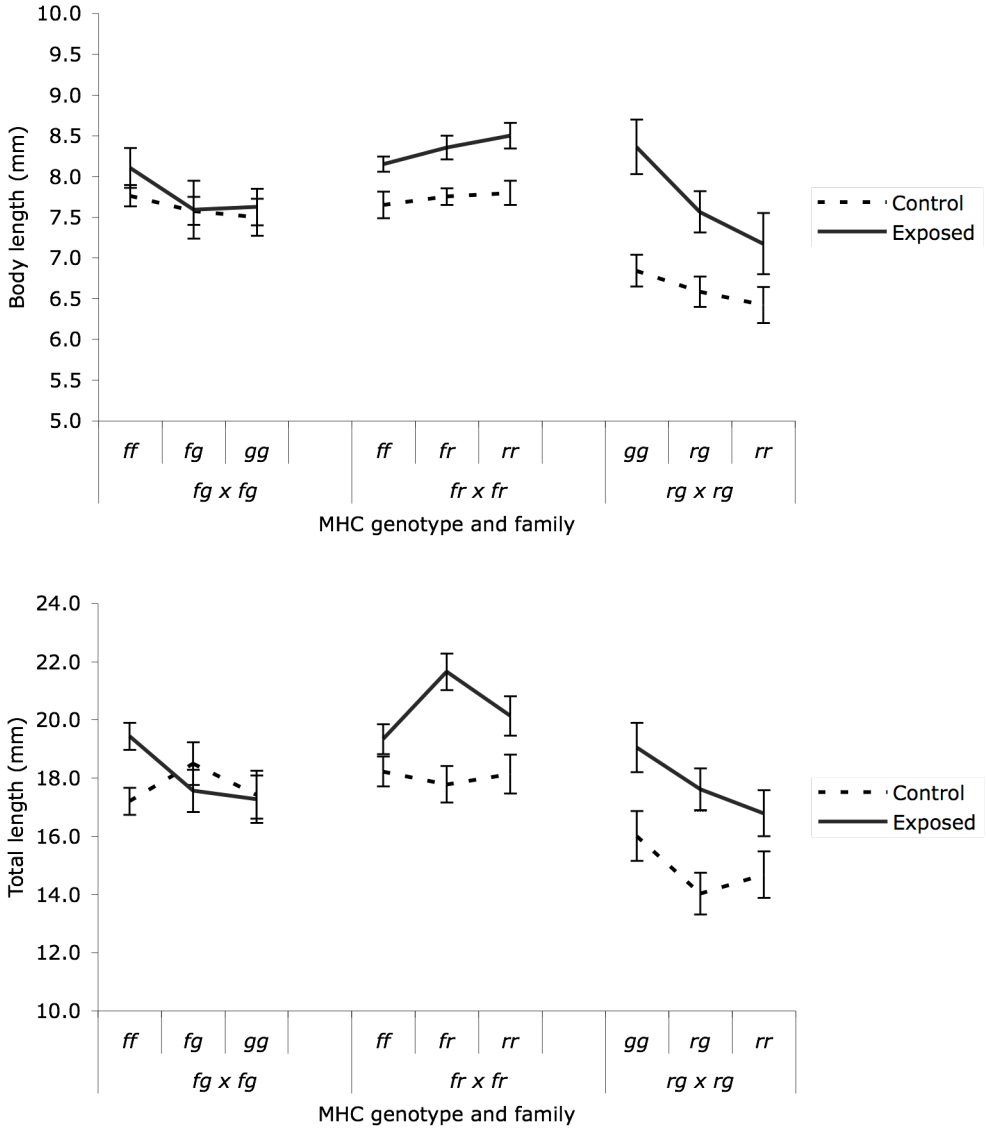


Figure 3.7 Body and total length (± SE) of tadpoles on day 18 with different MHC genotypes that were either exposed to live *A. hydrophila* or dead *A. hydrophila* as a control.

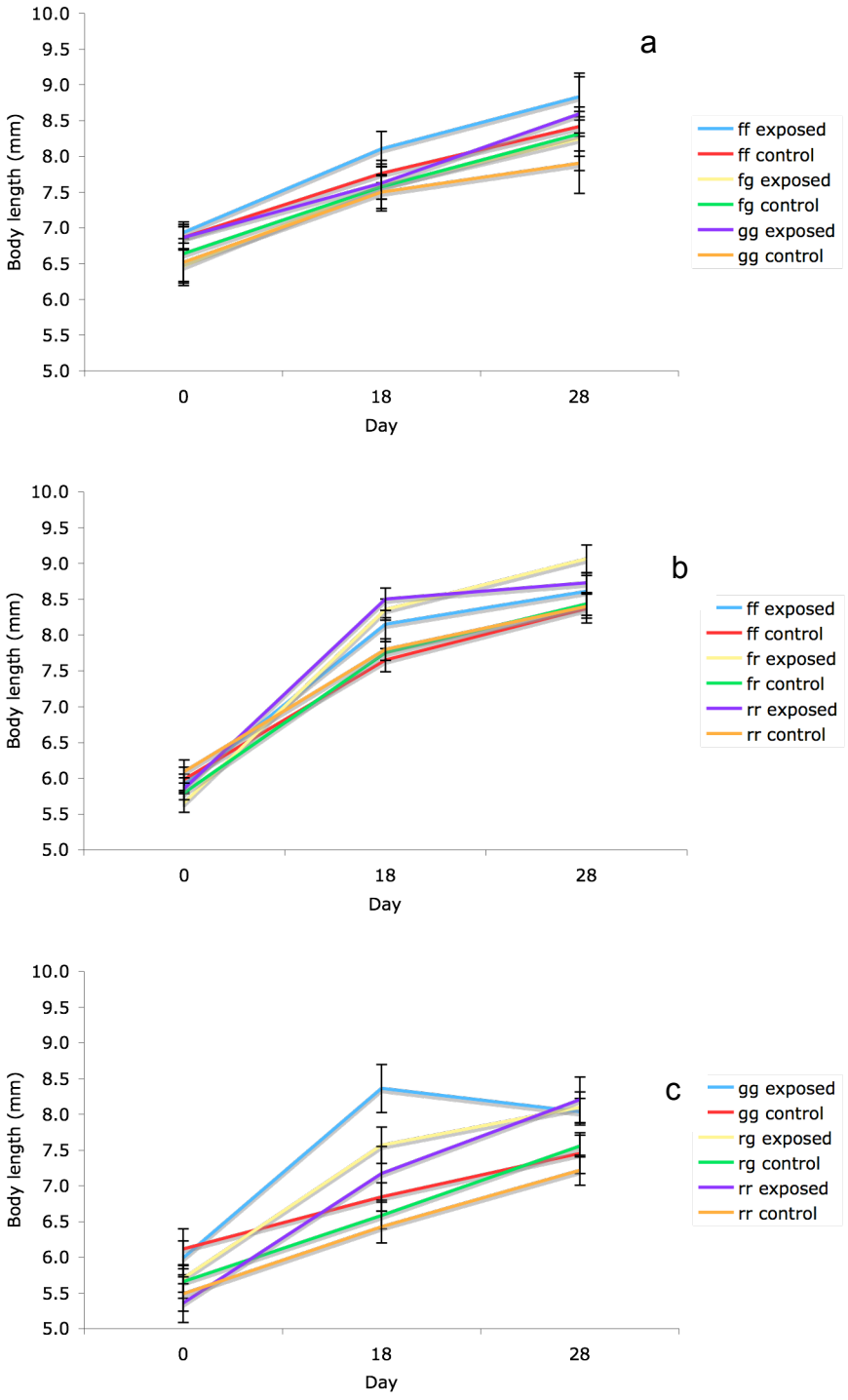


Figure 3.8 Body length (± SE) across time of tadpoles with each MHC genotype exposed to the live bacteria (exposed) or dead bacteria (control) in the three crosses (a) *fg* x *fg*, (b) *fr* x *fr*, (c) *rg* x *rg*.

Mortality

Significantly more tadpoles exposed to live *A. hydrophila* died than tadpoles exposed to dead *A. hydrophila* (Fig. 3.9)(χ^2 = 5.53, 1 d.f., P = 0.019). Tadpoles from the different families also significantly differed in mortality (χ^2 = 23.47, 2 d.f., P < 0.0001). Although not significant, there was a strong trend indicating that MHC genotype affected tadpole mortality (Fig. 3.10)(χ^2 = 11.78, 6 d.f., P = 0.067). More *rr* than *gg* tadpoles died, but both of these MHC genotypes had high mortality rates, and *ff* tadpoles were relatively resistant to *A. hydrophila*. In each family heterozygote mortality was intermediate to the two MHC genotype homozygotes (Fig. 3.10). There was no significant interaction between exposure and MHC genotype (χ^2 = 4.08, 6 d.f., P = 0.67).

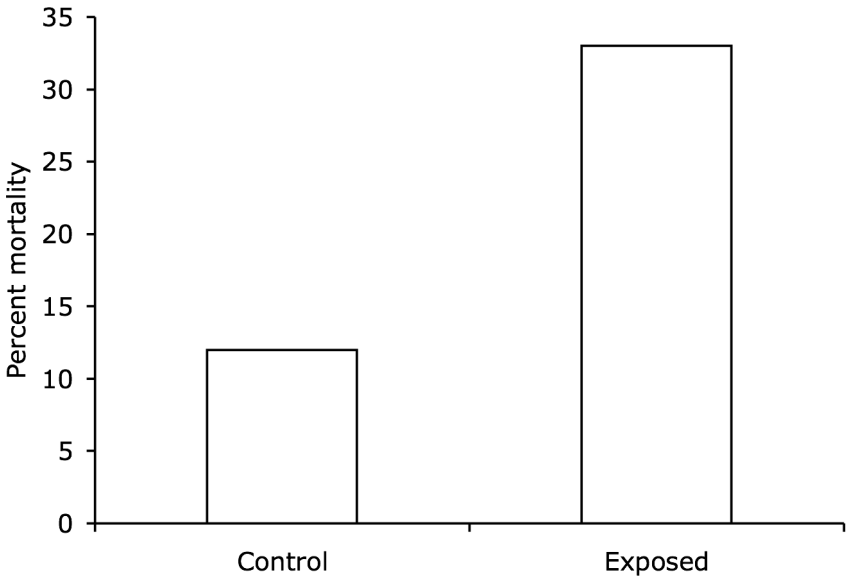


Figure 3.9 Mortality of tadpoles exposed to live (exposed) and dead (control) bacteria

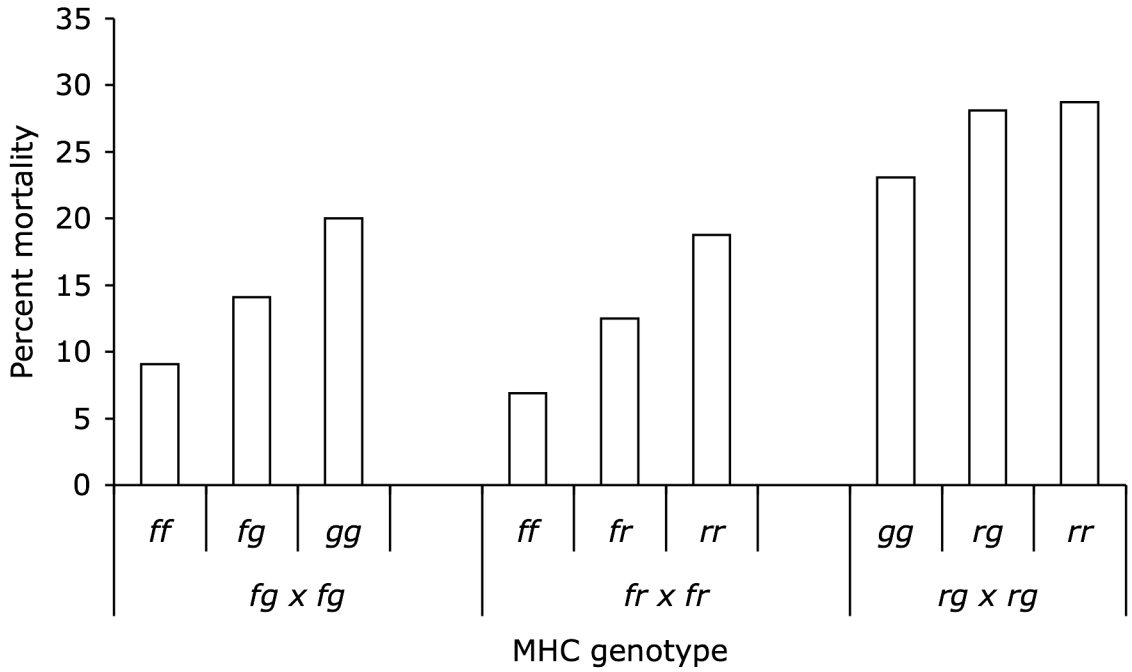


Figure 3.10 Mortality of tadpoles with each MHC genotype within each family.

Table 3.2 Summary of univariate ANOVA for the within-families experiment for body length and total length at days 0, 18 and 28.

		Day 0				Day 18				Day 28			
Body Length	DF	SS	MS	F	*P*	SS	MS	F	*P*	SS	MS	F	*P*
Family	2	23.623	11.811	23.91	<0.0001	10.58	5.29	11.4	<0.0001	21.49	10.74	15.11	<0.0001
Exposure	1	0.038	0.038	0.08	0.782	16.87	16.87	36.36	<0.0001	9.94	9.94	13.97	0.00026
Genotype(Family)	6	1.759	0.293	0.59	0.735	5.02	0.84	1.8	0.102	2.66	0.44	0.62	0.71
Genotype*Exposure(Family)	8	1.808	0.226	0.46	0.884	8.1	1.01	2.18	0.032	3.3	0.41	0.58	0.792
Error	159	78.559	0.494			73.78	0.46			113.05	0.71		
Total	176	106.181				113.01				150.31			
Total Length	DF	SS	MS	F	*P*	SS	MS	F	*P*	SS	MS	F	*P*
Family	2	344.5	172.25	66.28	<0.0001	90.24	45.12	6.185	0.0026	153.25	76.63	13.799	<0.0001
Exposure	1	0.93	0.93	0.36	0.55	181.15	181.15	24.832	<0.0001	30.62	30.62	5.514	0.02
Genotype(Family)	6	23.64	3.94	1.52	0.176	47.71	7.95	1.09	0.371	25.93	4.32	0.778	0.588
Genotype*Exposure(Family)	8	15.78	1.97	0.76	0.639	132.94	16.62	2.278	0.025	21.35	2.67	0.481	0.869
Error	159	413.22	2.6			1159.93	7.3			882.94	5.55		
Total	176	801.48				1615.54				1112.34			

Table 3.3 Summary of repeated-measures ANOVA results for the within-families experiment

	Body Length					Total Length			
Among Individuals	SS	DF	MS	*F*	*P*	SS	MS	*F*	*P*
Family	28.09	2	14.04	11.68	<0.0001	110.2	55.1	5.76	0.00385
Exposure	16.64	1	16.64	13.83	0.00028	132.8	132.8	13.88	0.00027
Genotype(Family)	6.2	6	1.03	0.86	0.527	75.3	12.5	1.31	0.255
Genotype*Exposure(Family)	6.32	8	0.79	0.66	0.729	78.9	9.9	1.03	0.415
Error	191.22	159	1.2			1521	9.6		
Within Individuals	SS	DF	MS	*F*	*P*	SS	MS	*F*	*P*
TIME	369.28	2	184.64	791.66	<0.0001	2251.7	1125.8	382.88	<0.0001
TIME*Family	27.6	4	6.9	29.59	<0.0001	477.8	119.5	40.63	<0.0001
TIME*Exposure	10.21	2	5.11	21.89	<0.0001	79.9	40	13.59	<0.0001
TIME*Genotype(Fam)	3.24	12	0.27	1.16	0.312	22	1.8	0.62	0.822
TIME*Genotype*Exposure(Fam)	6.9	16	0.43	1.85	0.0247	91.2	5.7	1.94	0.0169
Error	74.17	318	0.23			935.1	2.9		

Table 3.4 Summary of repeated-measures ANOVA of the within-families experiment comparing controls with dead bacteria and controls with clean bacterial media

	Body Length					Total Length			
Among Individuals	SS	DF	MS	*F*	*P*	SS	MS	*F*	*P*
Exposure	21.31	1	21.31	19.88	< 0.0001	79.99	79.99	8.00	0.0063
Error	64.33	60	1.07			599.63	9.99		
					H-F adj.				
Within Individuals	SS	DF	MS	*F*	*P*	SS	MS	*F*	*P*
TIME	63.50	2	31.75	157.72	<0.0001	398.48	199.24	58.79	<0.0001
TIME*Exposure	10.23	2	5.12	25.41	<0.0001	36.99	18.49	5.46	0.0054
Error	24.16	120	0.20			406.67	3.39		

Discussion

Among Families

Exposure to *A. hydrophila,* the tadpoles' MHC genotype and the egg deposition order all significantly affected survival and growth. Mortality increased with exposure dose. Tadpoles carrying the *g* haplotype were the most susceptible to this pathogen, whereas those carrying the *f* or *j* haplotype were more resistant. Heterozygotes between the susceptible haplotype, *g*, and a resistant haplotype, *f* or *j*, had mortality rates intermediate to the homozygotes of each haplotype. This complements the previous finding that the *f* haplotype conferred resistance and the *j* haplotype susceptibility to frog virus-3 (Gantress *et al.*, 2003). Furthermore, there is an interaction between MHC genotype and exposure dose that suggests that the MHC is involved in the resistance of tadpoles to this pathogen.

The response to pathogen exposure might influence the survival of the tadpoles with these different haplotypes. Tadpoles with *f* or *j* haplotypes reduced their growth when exposed to the pathogen and had high survival; tadpoles with the *g* haplotype did not appear to reduce their growth but had higher mortality.

Tadpoles developing from eggs that had been laid earlier in the evening were significantly smaller and more likely to die than those from the same parents deposited later in the evening. Intraclutch differences in size have been documented in several amphibian species (Crump 1984; Dziminski & Alford

2005), but never in *Xenopus*, nor has it been linked to time of ovipositioning. The cause of this difference is unclear.

I have found MHC haplotypes that confer resistance and susceptibility to a bacterial pathogen. I used MHC heterozygous tadpoles that were half-siblings of the MHC homozygous tadpoles (i.e. *fg* tadpoles are half-siblings of *ff* and *gg* tadpoles) from inbred strains to limit non-MHC heritable differences. However, heritable effects of non-MHC genes may still have had effects on the disease resistance of these tadpoles. I conducted the within family tests to control for non-MHC variation.

Within Families

As in the previous experiment, exposure to the pathogen significantly affected growth and decreased survival; furthermore, the impact of pathogen exposure was determined by the MHC genotype of the tadpoles (Fig. 3.7). These results indicate that the MHC affected the tadpoles' ability to deal with this pathogen after accounting for heritable non-MHC differences. While the effects of the MHC on mortality only approached significance the trends that were apparent in the 'among families' experiment were also present here. As before, *ff* tadpoles had low mortality and *gg* tadpoles had high mortality, as did *rr* tadpoles which were not tested in the previous experiment. Unfortunately I did not have sufficient adult frogs with the *j* haplotype to study its effect again.

I did not detect a significant effect of MHC genotype on susceptibility to pathogen-induced mortality in this experiment. This is likely due to the smaller

sample size and a change in methods. In this experiment tadpoles were isolated into individual beakers much earlier than in the previous experiment. This was necessary to genotype each tadpole but it accelerated these tadpoles' growth, and possibly their development, to a point where they were less susceptible to the pathogen than the tadpoles in the previous experiment. This may also explain why in the first experiment tadpoles exposed to the pathogen were smaller than the controls whereas in the second experiment tadpoles exposed to the pathogen were larger, and that a greater exposure dose was required to induce mortality.

The control tadpoles exposed to the dead bacteria were larger than the controls exposed to pelleted clean bacterial media. Tadpoles that survived exposure to this pathogen had access to an additional renewable food source whereas tadpoles exposed to the dead bacteria had an additional limited food source. Tadpoles exposed to pelleted clean bacterial media had no additional food source. This supports the use of dead bacteria as an appropriate control in the previous experiment.

General Discussion

Over all, I detected two MHC haplotypes that were resistant (*f* and *j*) and two that were susceptible (*r* and *g*) to *A. hydrophila*. Tadpoles that were MHC-heterozygous with both resistant and susceptible haplotypes experienced levels of mortality intermediate to the two MHC-homozygote types. The first experiment suggests that the MHC is important for resistance to this pathogen but was potentially limited by the non-MHC genetic effects that could not be

accounted for, despite my attempts to limit these effects by using half-siblings and inbred lines. My second experiment addressed this and the results further support my previous conclusion, that MHC genotype affects survival. Tadpoles with the *f* haplotype had far lower mortality rates than those with the *g* haplotype or the *r* haplotype. And those tadpoles with different MHC genotypes grew differently when challenged with *A. hydrophila*. Almost all genotypes grew more rapidly when exposed to the pathogen in the second experiment, but the degree of increased growth depended on the MHC genotype of the individual. MHC class II is expressed in high concentrations in the intestines of *X. laevis* (Liu *et al.* 2002). These findings suggest that tadpoles with greater resistance to *A. hydrophila* can use dangerous food sources better than siblings with weaker resistance.

The differences between these two experiments could be due to the changes in methodology necessitated by genotyping all the tadpoles and lower sample size in the second experiment. However, the consistency of the MHC effects including the higher resistance of tadpoles with the *f* haplotype, the susceptibility of tadpoles with the *g* haplotype, and the intermediate resistance of heterozygotes with resistant and susceptible haplotypes in both experiments provides evidence that the MHC, rather than other genes, influenced bacterial resistance in my experiments.

MHC diversity can be driven by selection for pathogen resistance or sexual selection. Pathogen-based selection can be through heterozygote advantage (Doherty & Zinkernagel 1975), in which heterozygous individuals benefit due

to their ability to detect a greater range of pathogens than either potential homozygotes. Or individuals may accrue benefit through rare allele advantage (Takahata & Nei 1990), in which pathogens evolve to avoid recognition by common MHC genotypes. Similarly, mating preferences can serve to maintain MHC polymorphisms by selecting mates with different MHC genotypes to increase the number of MHC heterozygous progeny (Hedrick 1992; Penn *et al.* 2002; Potts *et al.* 1994), or by providing a 'moving target' of extant MHC polymorphisms for rapidly evolving parasites that escape MHC-dependant immune recognition (the Red Queen hypothesis) (Ebert & Hamilton 1996; Penn & Potts 1999). Alternatively, because the ability to discriminate based on the MHC may facilitate kin discrimination, MHC-disassortative mating may have been selected to avoid the deleterious effects of inbreeding by facilitating mating with non-kin (inbreeding avoidance hypothesis) (Brown & Eklund 1994; Potts *et al.* 1994; Waldman 1987, 1988).

Previous work has shown specific MHC haplotype-specific resistance of *Xenopus laevis* to infection by frog virus 3 (Gantress *et al.* 2003), using animals with the same MHC haplotypes as described in this study. When exposed to this virus, significantly more individuals with the *j* haplotype died than with the *f* haplotype, and the animals with the *j* haplotype took twice as long to clear the infection (Gantress *et al.* 2003). MHC based allelic resistances and susceptibilities have been documented in many taxa including fish (Grimholt *et al.* 2003; Langefors *et al.* 2001; Lohm *et al.* 2002; Miller *et al.* 2004; Pitcher & Neff 2006; Wedekind *et al.* 2004), mice (McClelland *et al.* 2003; Nauciel *et al.* 1988), birds (Briles *et al.* 1977;

Westerdahl *et al.* 2005) and humans (Carrington *et al.* 1999; Hill *et al.* 1991; Tamonza *et al.* 2002). These specific allelic resistances can result in either heterozygote advantage or rare allele advantage.

Most commonly, animals that are heterozygous at the MHC have disease resistance intermediate to the two homozygous haplotypes (Penn *et al.* 2002), but over a lifetime of sequential infections or during co-infection, heterozygotes may have superior resistance to either homozygote haplotype (McClelland *et al.* 2003). Conversely, individuals with common MHC haplotypes may be more susceptible to pathogens that evolve to avoid these, thereby increasing the relative fitness of rare haplotypes (Langefors *et al.* 2001; Pitcher & Neff 2006). Both processes could drive MHC diversity, and support the increased fitness of MHC heterozygotes in a dynamic environment (for reviews see Apanius *et al.* 1997; Bernatchez & Landry 2003; Piertney & Oliver 2006).

Recent work shows that *X. laevis* tadpoles preferentially associate with tadpoles with which they share MHC alleles (Villinger 2007), although there are two striking deviations to this preference. Tadpoles with *rr* or *gg* genotypes actively avoid MHC identical tadpoles. These susceptible individuals might avoid MHC similar tadpoles to reduce their exposure to this ubiquitous pathogen.

Tadpoles that developed from eggs that had been laid earlier in the evening were significantly smaller and more likely to die than those from the same

parents that had been deposited later in the evening. The ecological significance of ovum size variability in growth and survival in X. laevis is unclear but has been described in other amphibians where females 'hedge their bets' on the environmental stability of breeding ponds (Crump 1984; Dziminski & Alford 2005; Dziminski & Roberts 2006; Kaplan 1980, 1989, 1992; Parichy & Kaplan 1992, 1995; Smith 1999; Tejedo & Reques 1992b). Well-provisioned embryos would survive under environmentally stressed conditions in which poorly provisioned eggs would fail to develop. The value of provisioning to eggs may extend beyond the risk of desiccation (Crump 1981, 1984; Kaplan & Cooper 1984) to those associated with pathogen diversity or density in breeding ponds. Nonetheless, these results might represent a laboratory artefact, for example, if females deposit older eggs first after being induced to oviposit.

Extensive die-off events of amphibians have been described across the world. Survivors of mass mortality events will be subject to the compounding pressures of increased inbreeding, further loss of genetic variation, and the risk of pathogen-induced extinction if a new or recently mutated pathogen evades immune recognition in these genetically depauperate groups (Acevedo-Whitehouse *et al.* 2003; Acevedo-Whitehouse *et al.* 2005; Pearman *et al.* 2004; Waldman & Tocher 1998). I have presented evidence for specific MHC haplotype based resistance and susceptibility to a common, if opportunistic, amphibian pathogen. Knowledge of specific resistances conferred by different genotypes may be critical to the success of captive rearing programmes (Mendelson *et al.* 2006). Moreover, the intermediate

susceptibility of MHC heterozygotes to either of their potential homozygote states reinforces the importance of maintaining MHC-diverse populations if amphibians are to survive exposure to new and changing pathogens.

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CHAPTER 4: INCREASED MORTALITY AND DEVELOPMENT IN RESPONSE TO NATURAL LEVELS OF FOREIGN MICROORGANISMS IN AFRICAN CLAWED FROG TADPOLES: BETTER THE DEVIL YOU KNOW, BUT NOT TOO WELL

Abstract

The major histocompatibility complex is the highly polymorphic gene structure responsible for self/non-self recognition for the immune system and has been linked to social behaviour in many taxa. Here I describe the results of an experiment in which I exposed *Xenopus laevis* tadpoles to natural doses of the microorganisms associated with frogs of different MHC genotypes. There were two alternative hypotheses. Tadpoles exposed to water from adult conspecifics with which they shared MHC haplotypes may be more susceptible than those exposed to water from MHC-dissimilar adult conspecifics, as pathogens may be adapted to and avoid recognition by the shared haplotypes. Alternatively, tadpoles may be more resistant to pathogens from MHC-similar frogs than those from MHC-dissimilar frogs as their shared MHC haplotypes have evolved to detect and defend against these familiar pathogens. I found that tadpoles exposed to water from MHC dissimilar animals developed more rapidly, yet without increased growth, and were more likely to die than those exposed to water from MHC-similar animals. I show that there is an optimal difference between the tadpoles' and the donors' MHC where tadpoles may be sufficiently different to the donor to

avoid its locally adapted pathogens, and sufficiently similar to not be exposed to especially virulent foreign pathogens.

Introduction

The major histocompatibility complex (MHC) underpins immunological self/non-self recognition, as well as social behaviour including mate choice (Eklund *et al.* 1991; Landry *et al.* 2001; Penn & Potts 1999; Piertney & Oliver 2006; Reusch *et al.* 2001; Thornhill *et al.* 2003). Extreme polymorphism at the MHC has been attributed to sexual or pathogen-based selection (reviewed in Piertney & Oliver 2006). As MHC alleles are codominant, individuals that are heterozygous at the MHC should be able to recognise a greater diversity of pathogens than those that are homozygous (Doherty & Zinkernagel 1975). Variation at the MHC also may be maintained by the advantage of having a rare genotype to which parasites have not adapted (Takahata & Nei 1990). Disassortative mating by MHC genotype may maintain high levels of MHC polymorphism within populations and increase the MHC heterozygosity of offspring (Penn *et al*. 2002; Penn 2002; Potts & Wakeland 1990). Or MHC dissasortiative mating may provide a 'moving target' of extant MHC polymorphisms for rapidly evolving parasites that escape MHC-dependent immune recognition (Red Queen hypothesis) (Bell 1985; Ebert & Hamilton 1996; Hamilton 1980; Hamilton *et al.* 1990; Levin 1975; Penn & Potts 1999). Finally, because the MHC is so polymorphic it may serve as a reliable kinship cue allowing inbreeding avoidance (Brown & Eklund 1994; Potts *et al.* 1994; Waldman 1987, 1988). None of these hypotheses are mutually exclusive.

African clawed frogs (*Xenopus*) and axolotls (*Ambystoma mexicanum*) are the only amphibians for which the MHC has been described (Flajnik & Kasahara 2001; Sammut *et al*. 1999). In *Xenopus*, as in most vertebrates, the MHC class I and II loci are closely linked (Liu *et al*. 2002; Nonaka *et al*. 1997). *Xenopus* tadpoles express low quantities of MHC class I molecules only in epithelial tissues, such as gills, lungs and intestines (Salter-Cid *et al*. 1998), and class II molecules on B cells and antigen-presenting cells (Flajnik *et al*. 1987). Despite their limited MHC class I expression, tadpoles are immunocompetent, although they are markedly more susceptible to viral infections than are adults (Gantress *et al*. 2003).

As *Xenopus* adults are entirely aquatic they share the same habitat as *Xenopus* larvae. Both adults and larvae are prone to infection by the same pathogens (Gantress *et al*. 2003), so vertical transmission of pathogens may be high. *Xenopus* adults also frequently cannibalise tadpoles (Measey 1998; Parker *et al.* 1947) which can also increase the rate of pathogen transfer (Pfennig *et al.* 1998).

Because of their short generation time, pathogens are presumed to have an advantage in the coevolutionary arms race with their hosts (Hamilton 1980; Hamilton *et al.* 1990). Pathogens can then evolve to better infect their local hosts than foreign hosts (pathogen local adaptation). Alternatively, hosts may be better able to resist their familiar pathogens than foreign pathogens (pathogen local maladaptation); this is more likely when host migration rate is high (Kaltz *et al.* 1999; Lively 1999; Oppliger *et al.* 1999). Local adaptation of

pathogens to their hosts is thought to be a common but complex phenomenon (Kaltz & Shykoff 1998; Lively 1999; Morgan *et al*. 2005; Nuismer 2006). The detection of local adaptation can be difficult, and may be dependent on the stage of host-parasite coevolution among other factors.

Here I tested whether tadpoles' growth, development and survival were differently affected by the natural microbial fauna of conspecific adults based on the MHC similarity between tadpoles and adults. I measured tadpole growth and development as they are linked to size at metamorphosis, post-metamorphic survival, and reproductive success (reviewed in Alford 1999; Berven 1990; Brockelman 1969). I exposed tadpoles to water in which these conspecifics had been housed. If the microorganims were locally adapted to their hosts' genetic background, then tadpoles that were exposed to water from adult conspecifics with which they shared MHC haplotypes would be more susceptible than those exposed to water from MHC-dissimilar adult conspecifics. Alternatively, tadpoles may be more resistant to pathogens from MHC-similar frogs than to those from MHC-dissimilar frogs if their shared MHC haplotypes have evolved mechanisms to resist infection by these familiar pathogens.

Materials & Methods

Six adult *Xenopus laevis* homozygous at the MHC (*ff, gg, jj*) were bred twice during one night and produced tadpoles bearing six genotypes (*ff, fg, fj, gg, gj, jj*). On the day of breeding, between 13:00 and 15:00, I isolated and primed

females by injection with 0.03 mg Luteinizing Hormone – Releasing Hormone (LH-RH; Argent Chemical Laboratories, Redmont, WA, USA) dissolved in 150 µL of sterilized water. I monitored the cloacae of the frogs from 5 to 8 h after priming. Once the cloacae displayed swelling and red colouration from increased blood flow, I injected the females with an additional 0.1 mg LH-RH dissolved in 500 µL of sterilized water and placed them into breeding tanks with plastic grates anchored by rocks to allow fertilised eggs to fall through and avoid contact with and damage by the breeding pair. I placed male frogs into the breeding tank with the female after they were injected with 0.03 mg LH-RH dissolved in 150 µL of sterilized water. I paired homozygotes of the same MHC type, and midway through their oviposition, I separated the amplexed pairs and placed them with partners with which they differed in MHC type.

After breeding, I separated the females into clean 60 L polyethylene tanks with continuous through-flow of filtered and aerated deep aquifer water at 21°C. I also placed two additional MHC-heterozygous females (*fg*, *gj*) into similar tanks. I fed the adults sliced ox liver every two days. Subsequently, I stopped water flow through the tanks to allow microorganisms to accumulate, and I concurrently stopped feeding the frogs to limit fouling.

Ten days later, I plated 10 µL of the tank water from each female's tank in triplicate onto tryptone soya agar (TSA, Oxoid, Basingstoke, UK), incubated the plates at 32°C, and counted the number of colonies at 24 and 48 h. I then

diluted the water to match to the lowest number of colonies found in the adults' water (from the *gg* female, 250 colony forming units/ml).

Two weeks after oviposition, I individually transferred 360 tadpoles, 60 of each genotype, from the breeding tanks each into a 1 L polypropylene beaker. I arranged the tadpoles into 6 blocks containing 6 full replicates. Every genotype was exposed to water preconditioned by every genotype of frog (*ff, fg, gg, gj, jj*) and clean water as a control. I fed tadpoles every 2 days with ground nettle suspension, and I provided increased quantities of food as the tadpoles grew. I added clean water every fourth day for 21 days to compensate for evaporation, and I moved each beaker one place each day to limit position effects.

After 21 days I concluded the experiment. I staged (Nieuwkoop & Faber 1956) and digitally photographed each tadpole from directly above (Nikon Coolpix 4500, with 1024 x 768 pixel images). I measured tadpoles' snout-vent length (SVL) and total length (TL) from the images using NIH ImageJ (v 1.3, National Institutes of Health, Bethesda MD, USA).

The length (SVL and TL) and stage data were analysed with multivariate general linear models (Statistica 6.1, Statsoft, Tulsa, OK, USA) with block, MHC genotype of the tadpoles and the exposure condition as fixed factors. I included orthogonal contrasts to compare the tadpoles exposed to the control water to all the experimentally exposed tadpoles. I contrasted the length and development of tadpoles exposed to their mothers' water to those exposed to

water from unrelated individuals to test whether there was different growth and development patterns based on exposure to kinship cues. I also analysed the same data with the number of alleles shared between the tadpoles and the adults that preconditioned the exposure water and block as fixed factors (the clean water exposure was excluded from this analysis), and the degree of similarity at the peptide binding region (PBR) of the MHC class I, II and combined. I calculated the percent amino acids that were shared between tadpoles and the adults that preconditioned the exposure water using published sequences (Flajnik *et al*. 1999; Liu *et al*. 2002). I used block and the degree of similarity at the class I, class II, and combined as fixed factors in three multivariate GLMs. I also analysed the relationship between the degree of similarity at the MHC class I, class II and combined on development by linear and quadratic regressions. I analysed the mortality data with a fully factorial binomial generalized linear model (GLZ) with the same fixed factors using a Logit Link function to create maximum-likelihood ratio estimates (Statistica 6.1).

Results

The tadpole's MHC genotype, the source of the exposure water, and the number of haplotypes shared between the tadpole and the donor water all affected the growth and development of the tadpoles. The growth and development of tadpoles differed significantly depending on the genotype of the frog with which the exposure water was conditioned (Fig. 4.1, 4.2, Table 4.1, Stage: $F_{5, 312} = 6.00$, $P < 0.0001$; SVL: $F_{5, 312} = 5.29$, $P = 0.00011$; TL: $F_{5,}$

$_{312}$ = 2.07, *P* = 0.069). Tadpoles that were exposed to the control water were not larger (orthogonal contrasts, SVL: $F_{1, 312}$ = 0.029, *P* = 0.87, TL: $F_{1, 312}$ = 1.26, *P* = 0.26) or more developmentally advanced (Stage: $F_{1, 312}$ = 1.52, *P* = 0.22) than those exposed to the preconditioned water (controls, Stage 47.73 ± 0.11, TL 16.52 mm ± 0.26 mm, SVL 6.92 mm ± 0.10 mm; exposed, Stage 47.62 ± 0.05, TL 16.09 mm ± 0.12 mm, SVL 6.85 mm ± 0.04 mm).

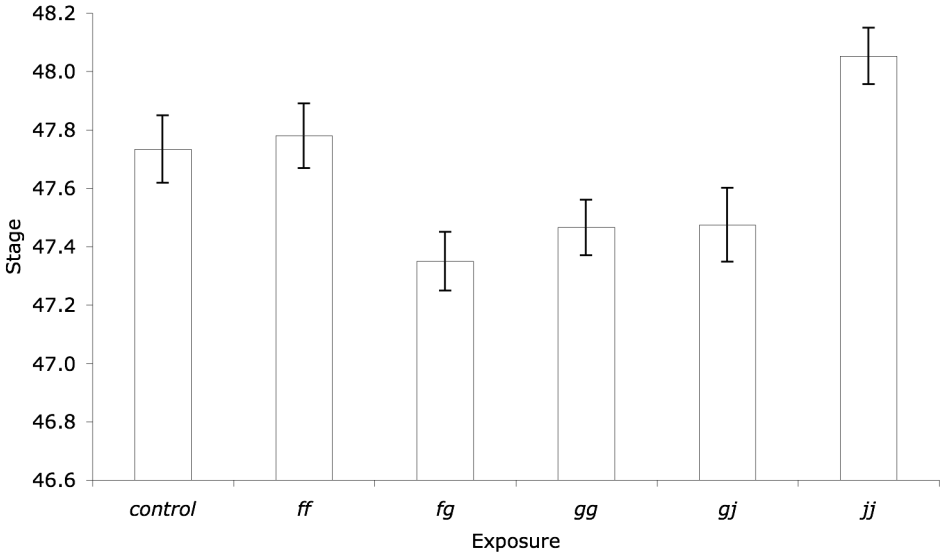


Figure 4.1 Mean developmental stage (±SE) of tadpoles exposed to water preconditioned by adult frogs with different MHC genotypes or clean water (control).

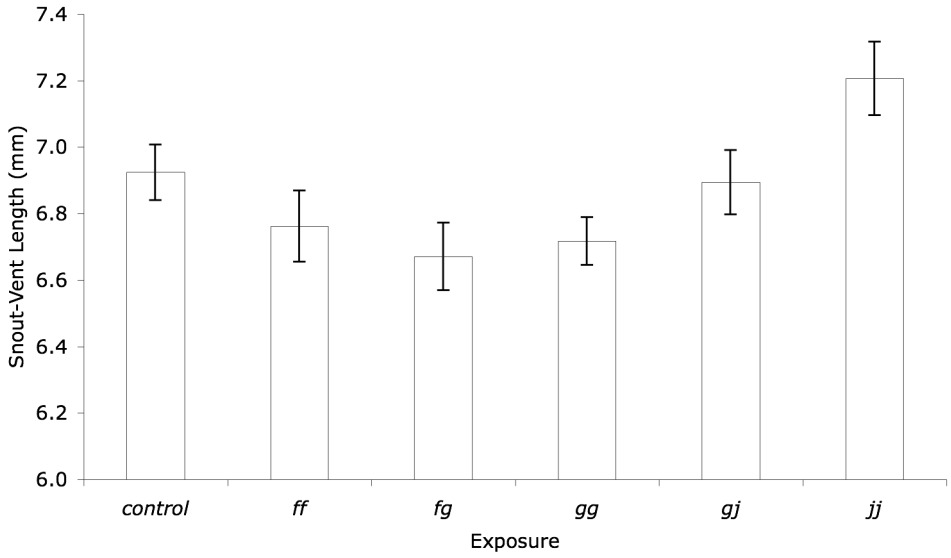


Figure 4.2 Mean Snout-Vent length (±SE) of tadpoles exposed to water preconditioned by adult frogs with different MHC genotypes or clean water (control)

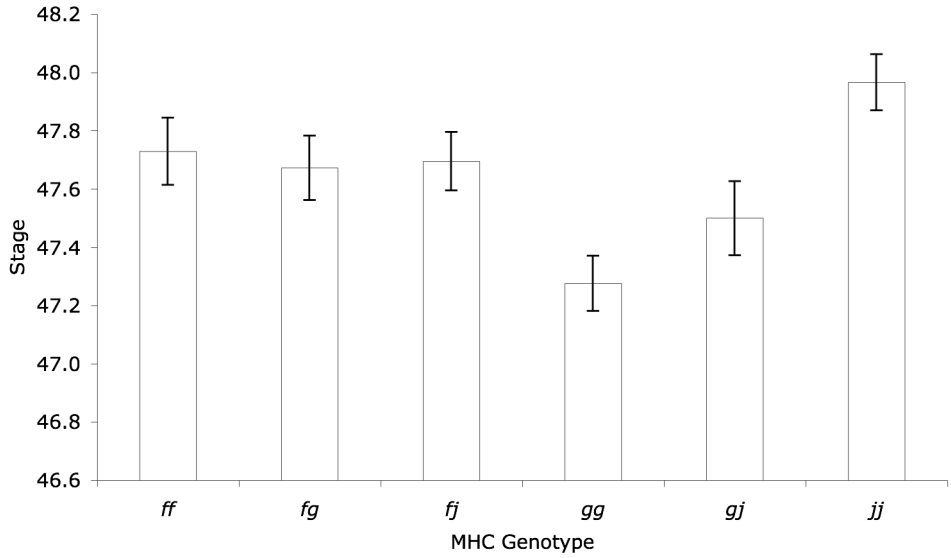


Figure 4.3 Mean developmental stage (±SE) of tadpoles of different MHC genotypes across all exposures.

Tadpoles with different MHC genotypes developed at significantly different rates (Fig. 4.3, $F_{5, 312} = 5.00$, $P < 0.0001$). But tadpoles with different MHC genotypes were not significantly different in length (SVL: $F_{5, 312} = 2.17$, $P = 0.057$; TL: $F_{5, 312} = 1.32$, $P = 0.26$). Developmental stage, but not growth, differed significantly dependent on the number of haplotypes the tadpoles shared with the source of the conditioned water (Fig. 4.4, $F_{3, 344} = 4.0$, $P = 0.01$). Developmental stage indicates how close tadpoles are to metamorphosis independent of growth. Tadpoles' size was not significantly affected by the number of haplotypes they shared with the donor's water (SVL: $F_{3, 344} = 0.09$, $P = 0.96$; TL: $F_{3, 344} = 0.97$, $P = 0.41$). Control tadpoles and those exposed to water whose donor shared no haplotypes with them had the highest developmental stage. Replicate blocks differed significantly in growth and development (Table 4.1). Tadpoles that were exposed to water preconditioned by their mothers were not significantly different in size (TL: 15.98 ± 0.27 mm, SVL: 6.76 ± 0.10 mm) or development (47.66 ± 0.11) than tadpoles exposed to water from unrelated females (TL 16.13 ± 0.14 mm: $F_{1, 312} = 0.10$, $P = 0.75$; SVL 6.87 ± 0.05 mm: $F_{1, 312} = 1.45$, $P = 0.23$; stage 47.61 ± 0.06: $F_{1, 312} = 0.18$, $P = 0.67$).

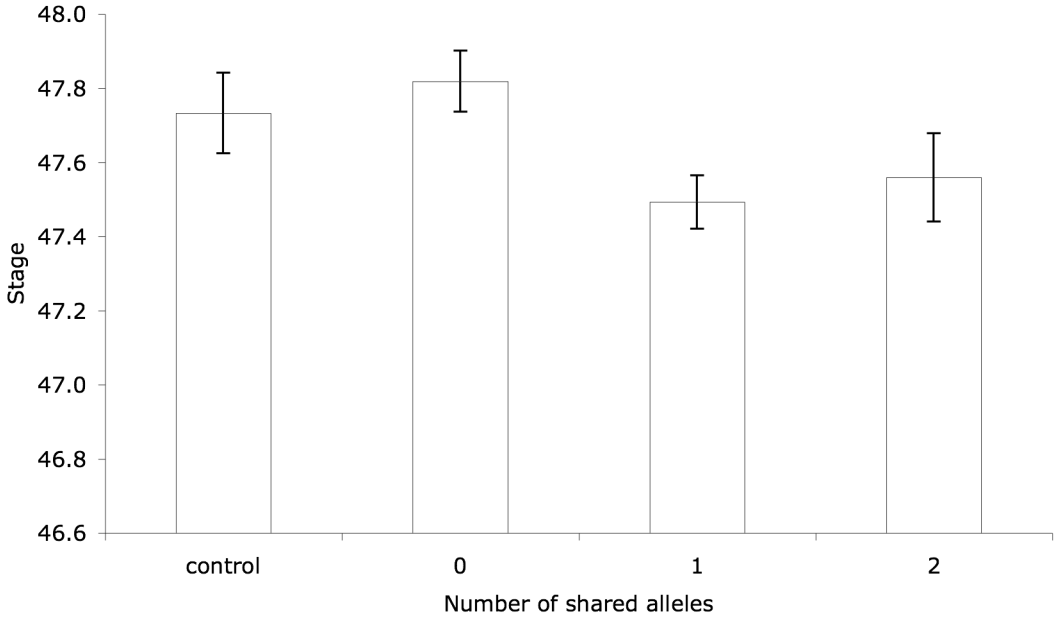


Figure 4.4 Mean developmental stage (±SE) of tadpoles exposed to water preconditioned by adults with which they shared 0 to 2 alleles and clean water as the control.

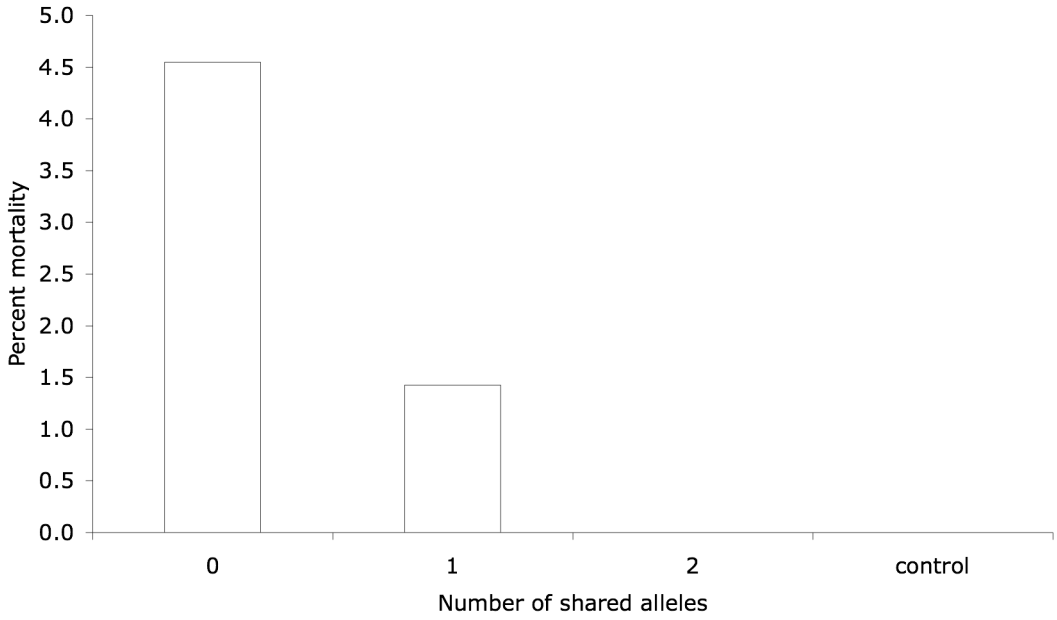
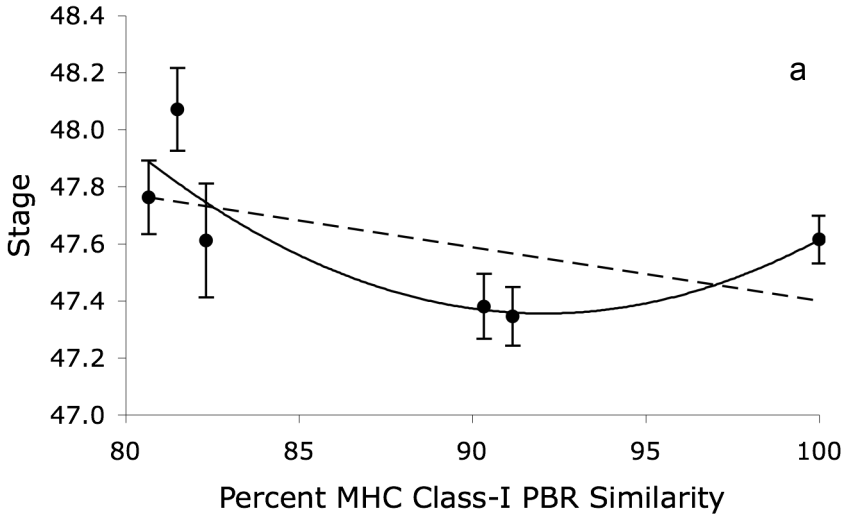
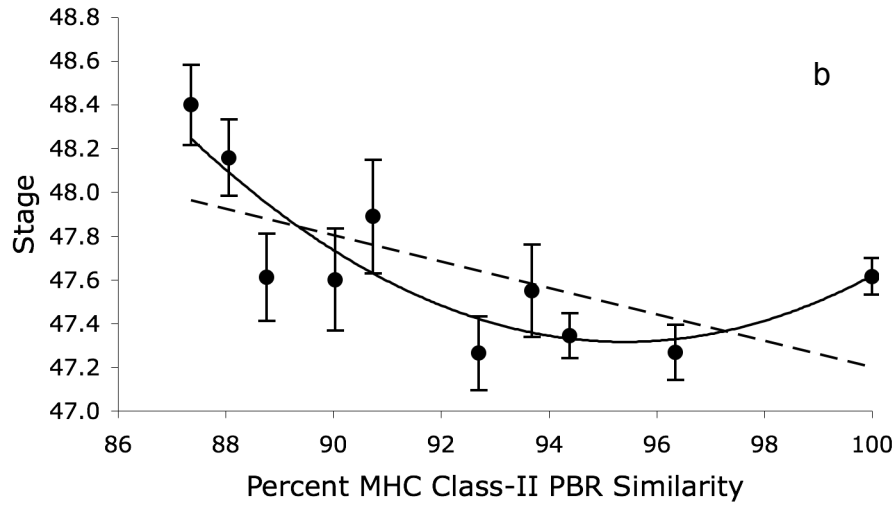


Figure 4.5 Percent mortality of tadpoles exposed to water preconditioned by adults with which they shared 0 to 2 alleles and clean water as the control.

Mortality was highest among tadpoles exposed to water from source frogs with which they shared no haplotypes, and decreased when the tadpoles and the water source frogs were increasingly similar (Fig. 4.5, Table 4.2, $\chi^2 = 8.07$, 1 d.f., $P = 0.0045$). The percent similarity between tadpoles and the exposure water's donor at the PBR significantly affected the tadpole development (class-I: $F_{1, 285} = 4.20$, $P = 0.041$, class-II: $F_{1, 285} = 9.43$, $P = 0.0023$, and combined: $F_{1, 285} = 6.85$, $P = 0.0093$). As the difference between the PBR of the tadpoles and the donor increased, so too did the rate of development (Table 4.1, Figs 4.6a-c). Tadpole development was significantly predicted by the degree of PBR similarity between the tadpoles and the donor for MHC-class II ($r^2 = 0.0265$, $P = 0.0052$) and class I and class II combined ($r^2 = 0.0182$, $P = 0.021$) when using linear regressions, but not for class I ($r^2 = 0.01$, $P = 0.087$). However, all of these analyses significantly fit quadratic regressions (Fig. 4.6a-c; class I Adj $r^2 = 0.0343$, $P = 0.0023$; class II Adj $r^2 = 0.0940$, $P < 0.0001$; combined Adj $r^2 = 0.0799$, $P < 0.0001$). PBR similarity was not significantly associated with either measure of length (Table 4.1) or with mortality (Table 4.2).


a
Stage
48.4
48.2
48.0
47.8
47.6
47.4
47.2
47.0
80
85
90
95
100
Percent MHC Class-I PBR Similarity


b
Stage
48.8
48.6
48.4
48.2
48.0
47.8
47.6
47.4
47.2
47.0
86
88
90
92
94
96
98
100
Percent MHC Class-II PBR Similarity

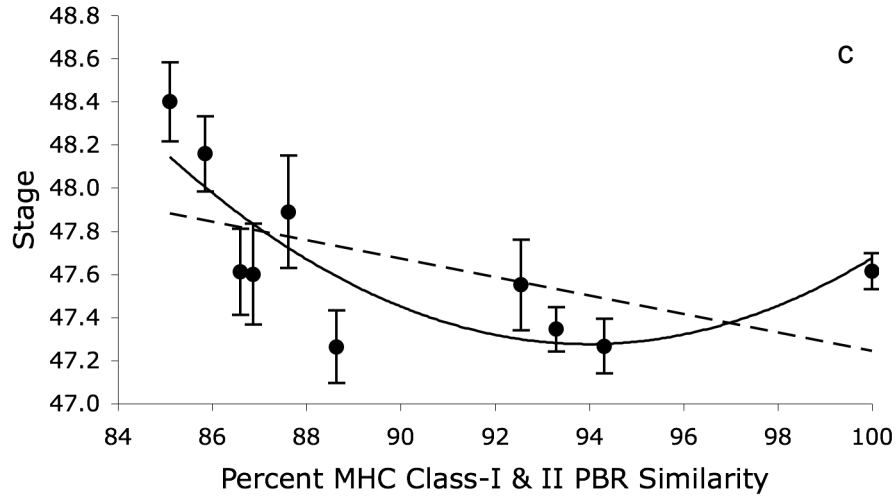

c
Stage
48.8
48.6
48.4
48.2
48.0
47.8
47.6
47.4
47.2
47.0
84
86
88
90
92
94
96
98
100
Percent MHC Class-I & II PBR Similarity

Figure 4.6 Mean developmental stage (±SE) of tadpoles exposed to water preconditioned by adults with which they shared different degree of MHC PBR similarity at the class-I (a), class-II (b), and combined (c). Solid lines are quadratic regressions (a) Adj $r^2 = 0.0343$, $P = 0.0023$, (b) Adj $r^2 = 0.0940$, $P < 0.0001$, (c) Adj $r^2 = 0.0799$, $P < 0.0001$, dashed lines are linear regressions (a) Adj $r^2 = 0.0066$, $P = 0.0873$, (b) Adj $r^2 = 0.0232$, $P = 0.0052$, (c) Adj $r^2 = 0.0148$, $P = 0.0209$.

Table 4.1 Summary of ANOVA results of MHC, number of shared haplotypes, and degree of similarity at the PBR of MHC class-I, class-II, and combined on tadpole development and growth

		Stage				Snout-Vent Length				Total Length			
	d.f.	SS	MS	F	*P*	SS	MS	F	*P*	SS	MS	F	*P*
Block	5	14.70	2.90	5.0	0.00024	22.14	4.43	9.30	< 0.0001	122.69	24.54	6.27	< 0.0001
Exposure	5	18.90	3.80	6.0	<0.0001	12.59	2.52	5.29	0.00011	40.44	8.09	2.07	0.069
MHC	5	16.10	3.20	5.0	<0.0001	5.17	1.03	2.17	0.057	25.79	5.16	1.32	0.26
Exposure*MHC	25	16.50	0.70	1.0	0.34	14.87	0.59	1.25	0.19	73.71	2.95	0.75	0.80
Error	312	187.20	0.60			148.65	0.48			1221.22	3.91		
Total	352	255.30				200.28				1475.44			
Block	5	16.20	3.20	4.70	0.00038	15.24	3.05	5.45	0.000084	87.72	17.54	4.3	0.00087
No. Shared Alleles	2	6.80	3.40	4.90	0.0079	0.1	0.05	0.09	0.92	4.8	2.4	0.59	0.56
Error	285	196.20	0.70			159.45	0.56			1163.37	4.08		
Total	292	218.90				175.14				1252.87			
Intercept	1	5356.12	5356.12	7645.27	<0.0001	95.87	95.87	172.16	< 0.0001	557.60	557.60	136.56	< 0.0001
Block	5	16.39	3.28	4.67	0.00040	15.77	3.15	5.66	< 0.0001	84.80	16.96	4.15	0.0012
Shared PBR Class-I	1	2.58	2.58	3.68	0.056	0.28	0.28	0.50	0.48	0.37	0.37	0.09	0.76
Error	286	200.37	0.70			159.27	0.56			1167.80	4.08		
Total	292	218.95				175.14				1252.87			
Block	5	16.09	3.22	4.67	0.00041	15.64	3.13	5.61	< 0.0001	84.69	16.94	4.15	0.0012
Shared PBR Class-II	1	5.90	5.90	8.56	0.0037	0.07	0.07	0.12	0.73	0.01	0.01	0.00	0.96
Error	286	197.05	0.69			159.48	0.56			1168.16	4.08		
Total	292	218.95				175.14				1252.87			
Block	5	16.29	3.26	4.69	0.00039	15.69	3.14	5.63	< 0.0001	84.72	16.94	4.15	0.0012
Shared PBR Both	1	4.27	4.27	6.15	0.014	0.15	0.15	0.27	0.60	0.12	0.11	0.03	0.87
Error	286	198.68	0.70			159.39	0.56			1168.06	4.08		
Total	292	218.95				175.14				1252.87			

Table 4.2 Summary of binomial GLMs of the effects of MHC, the number of shared haplotypes, and degree of similarity at the PBR of MHC class-I, class-II, and combined on tadpole survival.

	d.f.	Log-Likelihood	χ^2	*P*
Block	5	-32.25	4.52	0.48
Exposure	4	-27.77	8.97	0.062
MHC	5	-25.73	4.09	0.54
Exposure*MHC	9	-17.80	15.86	0.070
Block	5	-32.25	4.52	0.48
No. Shared Alleles	1	-28.22	8.07	0.0045
Shared PBR Class-I	1	-33.104	0.24	0.63
Block	5	-30.483	5.24	0.39
Shared PBR Class-II	1	-33.222	0.0024	0.96
Block	5	-30.604	5.24	0.39
Shared PBR Both	1	-33.190	0.066	0.80
Block	5	-30.573	5.23	0.39

Discussion

Tadpoles' growth, development, and survival were significantly influenced by the degree of similarity between tadpoles' MHC genotype and local conspecifics. Tadpoles' development accelerated as the MHC differences between themselves and conspecifics increased. This did not correspond with increased growth, which suggests that it is a response to an unfavourable environment. When environmental conditions are poor tadpoles can accelerate their development to escape the unfavourable conditions (Alford 1999; Denver *et al*. 1998; Schmuck *et al*. 1994; Semlitsch & Caldwell 1982). Although adult *Xenopus* are in the same environment as the larvae, they may be better able to deal with environmental dangers.

Cannibalism is common in anurans (Crump 1983, 1990; Pfennig *et al*. 1998). *Xenopus* tadpoles are frequently cannibalised by adults (Measey 1998; Parker *et al*. 1947). Small frogs also may be eaten by conspecifics, but may be better able to avoid conspecific predation. My earlier work (Chapter 2) indicates that when tadpoles are reared with unrelated individuals the variation in growth within a group is increased. Those tadpoles that grow and develop rapidly will metamorphose sooner than their tank mates and may cannibalise their neighbours. The tadpoles in this study were kept individually with abundant food, but may have responded to the cues from the exposure water as though other unrelated individuals were nearby. Rapid development and metamorphosis would remove these tadpoles from competition with the unrelated tadpoles that are presumably nearby, and these potential competitors also may offer a valuable food source for the newly metamorphosed frog.

Metamorphosis also may provide an immunological refuge from pathogens. Three times as many tadpoles died when they were exposed to water from a donor with which they shared no haplotypes than water from frogs with which they shared one haplotype. These data suggest that foreign pathogens are more dangerous to these tadpoles than pathogens from MHC-similar animals. Adult *Xenopus* have a more complete immune system than do tadpoles as only adults have full expression of both MHC class-I and II molecules (Salter-Cid *et al.* 1988). Therefore, rapid metamorphosis may be advantageous to avoid pathogens that could kill a tadpole. This strategy is not without its risks, as the immune system is considerably inhibited during metamorphosis to

avoid autoimmune attacks on the newly created tissues (Flajnik *et al*. 1987; Horton *et al*. 1996). However, caution is warranted in interpreting the mortality data, as overall few individuals died.

My regression analyses indicate that there is an optimum MHC difference between tadpoles and the donor water. Tadpoles exposed to water from frogs that differed the most in their MHC-PBR accelerated their development, and as the similarity between the tadpoles and the water donors increased, the tadpoles' developmental rate reduced, until the donor and tadpole MHC were identical when tadpole development increased again (Fig. 4.6a-c). This trend is considerably stronger for class-II than class-I differences, which suggests that extracellular pathogens or parasites are involved. Tadpole length did not increase with development.

Tadpoles that were exposed to very different water may have been exposed to foreign pathogens with greater virulence to novel hosts. This impact was reduced as the similarity between the MHC-PBR of the tadpoles and the adult donors increased until they were identical. However, tadpoles exposed to water from MHC identical donors, that were more advanced in development than tadpoles exposed to water from donors with intermediate MHC-PBR similarity, may be prone to infection from their pathogens. The animals exposed to water from the intermediate, optimal region of MHC difference (90-96% similar) may be different enough from the donor not to be inherently susceptible to their pathogens, but similar enough that the donors would not expose them to highly virulent foreign pathogens. Wegner *et al*. (2003)

demonstrated that sticklebacks with an intermediate number of MHC class-IIb alleles were most resistant to challenges with multiple parasites. A similar process may be occurring in this case where, rather than an optimal number of alleles, individuals may be optimally different at the PBR to conspecifics in their environment. What is optimally different will change with the addition and removal of individuals bearing their own distinct faunas of pathogens and parasites.

Pathogens that are locally adapted can better infect their local hosts than foreign hosts, however, it is also possible for hosts to be better able to resist their familiar pathogens than foreign pathogens (pathogen local maladaptation), especially when host migration rate is high (Kaltz *et al.* 1999; Lively 1999; Oppliger *et al.* 1999). Locally adapted pathogens are predicted to infect a greater proportion of their sympatric hosts than allopatric hosts (Gandon *et al*. 1996; Kaltz & Shykoff 1998). In some host-pathogen systems, the resistance of one host can effectively exclude competitors from regions with the resistant host by high virulence to the alternative host (de Castro & Bolker 2005; Kaltz & Shykoff 1998; Schmitz & Nudds 1994). It then may be more beneficial for a host to maintain an infection by a common locally adapted pathogen that has low virulence than to exclude it and allow foreign more virulent pathogens an opportunity to infect it as antagonistic processes allow for both inter- and intraspecific competition between microorganisms (Atlas & Bartha 1998; Fredrickson & Stephanopoulos 1981; Lina *et al*. 2003; Sicard *et al*. 2006; Whipps 2001).

The role of the MHC in disease resistance has been studied extensively in many organisms, but rarely in amphibians. Gantress *et al*. (2003) found MHC-haplotypes that were associated with resistance or susceptibility to frog virus 3, and I previously described haplotypes that conferred resistance or susceptibility to the ubiquitous bacterial pathogen, *Aeromonas hydrophila* (Chapter 3). This is the first study that examines the role of natural MHC-specific variation on pathogen resistance. Further studies that examine the natural levels of MHC variation in *Xenopus laevis* and the microbial diversity in their environment would help elucidate the generality and ecological significance of MHC based immunity to ambient microorganisms.

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CHAPTER 5: THE FAUNA AND IMPORTANCE OF BACTERIA ASSOCIATED WITH NEW ZEALAND FROGS

Abstract

Despite their potential importance in amphibian disease, bacteria pathogenic to amphibians have received little attention since the identification of the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. Here I present an inventory of bacteria found in the gut and skin (non-systemic sites) and heart, muscle, and abdominal cavity (systemic sites) of captive frogs (*Litoria aurea, Litoria raniformis, Leiopelma archeyi, Leiopelma hochstetteri,* and *Xenopus laevis*) that died at two New Zealand universities. I found several species of bacteria previously identified as amphibian pathogens, including *Aeromonas hydrophila*, *Chryseobacterium meningosepticum*, *Enterobacter agglomerans*, *Flavobacterium indologenes*, *Pseudomonas aeruginosa* and *Stenatrophomonas maltophilia*. I also found many bacteria in systemic sites that have not been considered pathogenic to amphibians, including the most common isolate *Corynebacterium urealyticum*. None of the frogs tested positive for *B. dendrobatidis*. I discuss the potential importance of these species of bacteria as amphibian pathogens and as protective probiotics using New Zealand frogs as a case study.

Introduction

Amphibians have been declining worldwide since the 1960's (Carey 2000; Daszak *et al.* 1999; Houlahan *et al.* 2001; Waldman & Tocher 1999). Populations have disappeared both from compromised and pristine environments and on all continents inhabited by amphibians. Amphibian declines are most commonly attributed to infectious organisms such as iridoviruses (Cunningham *et al.* 1996; Daszak *et al.* 2003; Green *et al.* 2002) and the chytrid fungus *Batrachochytrium dendrobatidis* (Berger *et al.* 1998; Bosch *et al.* 2001; Burrowes *et al.* 2004; Lips *et al.* 2006, 2004; Mendelson *et al.* 2006; Rachowicz *et al.* 2006).

Before global amphibian declines became of concern, bacteria, in particular *Aeromonas hydrophila*, were considered the most likely cause for local population die-offs (Bradford 1991; Frye 1985; Nyman 1986; Russell 1898). *Aeromonas hydrophila* was identified as an important amphibian pathogen as early as the late nineteenth century (Russell 1898) and has since been consistently cultured from sick and dying frogs (Bradford 1991; Cunningham *et al.* 1996; Frye 1985; Mauel *et al.* 2002). Other bacteria that have been implicated in amphibian mortality, especially among captive populations, include *Chyseobacterium meningosepticum* (Green *et al.* 1999; Mauel *et al.* 2002), *Chlamydia pneumoniae* (Reed *et al.* 2000), and *Citrobacter freundii* (Mauel *et al*., 2002). Interest in the role of bacterial diseases in amphibian mortality has waned as attention has focused on *B. dendrobatidis* and iridoviruses as primary causes of global amphibian decline. Bacteria are now

considered by most to be secondary opportunistic pathogens of amphibians (Carey 2000; Cunningham *et al.* 1996; Hird *et al.* 1981).

Pathogens, even if regarded as secondary, potentially can become highly dangerous to amphibians, both in the laboratory and the field. Pesticides, temperature, and crowding can compromise the immune system of amphibians (Carey & Bryant 1995; Hayes *et al.* 2006; Taylor *et al.* 2001). More generally, any chronic stressors can cause immunosuppression and increase disease susceptibility (Goulson & Cory 1995; Keller *et al.* 1981; Pai *et al.* 1995; Rigney *et al.* 1978; Rollins-Smith & Cohen 2005; Taylor *et al.* 2001; Wedemeyer 1997). Pathogens also can increase in virulence when hosts are physiologically stressed. *Aeromonas hydrophila* cultures increase their growth rate when exposed to norepinephrine (Kinney *et al.* 1999), levels of which would be elevated in stressed individuals. Environmental stressors can make hosts vulnerable to common pathogens, which could lead to amphibian dieoffs.

The New Zealand frog fauna consists of three introduced species - *Litoria aurea* and *Litoria raniformis*, both classified threatened or endangered in Australia, and *Litoria ewingii,* which suffers localized declines in Australia - and three endemic, archaic species, *Leiopelma archeyi, Leiopelma hochstetteri,* and *Leiopelma hamiltoni* (Holyoake *et al.* 2001). *Leiopelma archeyi* have declined markedly in their main populations (Bell *et al*., 2004) and are threatened with extinction. Population declines in the introduced frogs and in *L. archeyi* may be associated with infection by *B. dendrobatadis* (Bell *et*

al. 2004; Waldman *et al.* 2001). As a first step to understanding amphibian morbidity and mortality, pathogens need to be identified and compared with the normal microfauna. I thus cultured bacteria from frogs that died in my facilities to determine the bacteria present.

Materials & Methods

New Zealand frogs (*Litoria* and *Leiopelma*) from the wild, from 1999 to 2004, were brought into the captive facility at the University of Canterbury. *Xenopus laevis* were obtained from collectors who captured them in the field in South Africa and others from captive breeding colonies. Additionally, some *Litoria aurea* were purchased from a commercial frog farm and were housed briefly at Massey University. Different methods were used at the two universities. I present results from both locations here.

Litoria raniformis and *Litoria aurea* were fed houseflies once a week. *Leiopelma archeyi* and *Leiopelma hochstetteri* were fed fruit flies with vestigial wings, wax worms, or small locusts once a week. In all cases sufficient food was added so that it was available for the whole week. All insect food was cultured on site. *Xenopus laevis* adults were fed wax worm larvae, thinly sliced ox liver, or Nutrafin turtle pellets (Rolf C. Hagen, Mansfield, Massachusetts, USA) twice a week.

I refrigerated frogs that died at 4°C, for no longer than 18 h, until culturing. I collected skin swabs from the ventral hind limbs and feet, forelegs, and/or

abdomen with sterilised cotton-buds. Swabs were inoculated aseptically into McCartney bottles of sterile broth media. Two non-selective broths were used: Luria broth (LB, Bio101, Carlsbad, California, USA) and tryptone soya broth (TSB, Oxoid, Basingstoke, UK). To test for systemic bacterial infections, I dissected the frogs under aseptic conditions, and cultured the heart in broth. Before the first incision I thoroughly swabbed the abdomen with 70% ethanol. I sterilized all dissecting instruments in 70% ethanol before and after the dermal incision, and before the heart was removed. I removed and preserved one forelimb at the carpel and one hind limb from the tarsus in 70% ethanol for polymerase chain-reaction (PCR) based detection of the chytrid fungus, *Batrachochytrium dendrobatidis*. The carcasses were preserved in 10% neutral-buffered formalin for histology.

All cultures were incubated aerobically for 24 ± 4 h at 32°C. The broth cultures were then plated onto tryptone soya agar (TSA, Oxoid, Basingstoke, UK) and incubated under the same conditions. Bacterial cultures were sent to the National Centre for Disease Investigation, Wallaceville (Biosecurity New Zealand, Ministry of Agriculture and Forestry) for phenotypic identification. Bacteria isolated from the muscle, heart, or abdominal-cavity were considered to be systemic infections. Bacteria found in the gastrointestinal tract or skin were considered non-systemic.

Sixty juvenile *L. aurea* (snout-vent length 22 – 30 mm) were purchased from a commercial frog supplier (New Zealand Frog Farms, Ardmore, Auckland, New Zealand). The firm exports frogs to the international pet trade, especially to

North America and Europe. The frogs were shipped overnight to Massey University, Palmerston North, and were fed houseflies, mealworms and aphids 2 to 3 times per week. Insects were cultured at Massey University or purchased from a commercial supplier (Biosuppliers, Auckland, New Zealand).

Within a few weeks, these frogs began to die. After the first deaths, surviving frogs were moved to sterile plastic containers but the remaining frogs died. Carcasses were sealed in airtight plastic bags (to prevent dehydration during storage) and stored at -20 °C. Necropsies were conducted including gross examination of animal condition, bacterial and histological investigations and PCR tests for chytrid fungus. Bacteria were cultured as previously described. Cultures were identified using three commercial aerobic bacterial identification systems (RapID CB plus and RapID NF plus, Remel, Lenexa, Kansas, USA; and Microbacter 24E (12E/12A + 12B, Medvet diagnostics, Thebarton, South Africa). Samples for PCR and histology were taken as for the University of Canterbury population.

All bacteria were assessed for pathogenic and anti-fungal properties. Bacteria were classed as being pathogenic if amphibian mortality previously had been attributed to them, or as possible pathogens of amphibians if they had been described as pathogens of fish and reptiles. Bacteria were considered as having anti-fungal attributes if they were described as such in the literature. Additionally, I conducted GenBank searches for the presence of chitinase genes in every species of bacteria identified. I selected chitinase as an

indication of anti-fungal activity as it is the most widely studied anti-fungal compound (Whipps 2001) and would be the most likely compound, and associated gene structure, to be described in the literature or GenBank. I classified bacteria bearing these genes as likely to have anti-fungal attributes.

Toe clips were tested for amphibian chytrid fungus by species-specific PCR. Ventral skin and/or skin from the foot of were examined for histological analysis. The samples were dehydrated, cleared with terpineol, and embedded in paraffin. Sections were microtomed at 5 µm and stained with Mayer's double strength haemalum and eosin. Some sections were stained with Ayoub-Shklar stain for signs of *B. dendrobatidis*. Identification of *B. dendrobatidis* was based on the diagnostic characters described by Berger *et al*. (1999).

I used primers (for 5' gacatggtagccagagcat 3', rev 5' gccttcgcaatagtttgtcc 3') based on the *B. dendrobatidis* genomic DNA nucleotide sequence of small subunit SSU rRNA in the BLAST nucleotide database (National Centre for Biotechnology and Information, Bethesda, Maryland, USA). These primers target a 218 bp region of the 18s SSU rRNA genomic sequence. The 25 µl PCR reaction comprised 0.5 µM forward and reverse primers, 1 x *Taq* PCR buffer, 2.0 mM $MgCl_2$, 200 µM dNTPs and 1 unit of *Taq* DNA polymerase (Roche). Amplification consisted of an initial 5 min denaturation at 94°C, followed by 30 cycles with denaturation at 94°C for 45 s, annealing at 54°C for 30 s and extension at 72°C for 45 s. After 30 cycles, the final extension was at 70°C for 7 minutes and analysed by electrophoresis on 2% agarose gels.

I used a positive control of DNA isolated from three sources. *B. dendrobatidis* isolated from: *Litoria raniformis*, *Litoria lesuari*, and *Bufo baxteri*. The primers for *B. dendrobatidis* failed to amplify DNA from *Aspergillus*, *Mucor* sp, *Saprolegnia*, or two other species of chytrid fungi: *Neocallimastix frontalis* and *Chytriomyces hyalinus*.

Results

Twenty-nine bacterial species were isolated from systemic sites (Table 5.1) and 33 from non-systemic sites (Table 5.2). Of these bacteria, 15 species were found in both systemic and non-systemic areas but often not in the same animal. The most common isolates were *Corynebacterium urealyticum* (isolated in 11 cases), *Myroides odoratum* (7), *Arcanobacterium haemolyticum* (5), and *Hafnia alvei* (5). Eight species of bacteria (*Acinetobacter haemolyticus*, *Aeromonas hydrophila*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Enterobacter agglomerans*, *Flavobacterium indologenes*, *Pseudomonas aeruginosa*, *Stenatrophomonas maltophilia*) from systemic sites are of clinical significance to amphibians (Table 5.3 and references therein). Of the systemic isolates 24 were gram negative and 5 were gram positive. Similarly 27 of the non-systemic isolates were gram negative and 4 were gram positive (excluding two isolates with unsatisfactory identification). No animals tested positive for *B. dendrobatidis* by PCR or histology. Based on literature and GenBank searches, 7 species of

bacteria found in systemic sites and 6 species from non-systemic sites, show evidence of antifungal properties (Table 5.4).

Table 5.1 Bacteria cultured from systemic sites of 71 frogs and their incidence. The sample size for each frog species is given in parentheses. Bacterial species marked with ^ were found in both systemic and non-systemic sites. Species marked with * are known amphibian pathogens, † species are known or suspected to have antifungal properties.

Bacteria	Isolates	% Frogs	Frog Species
Corynebacterium urealyticum^	11	15.5	*Litoria aurea* (11)
Myroides odoratus^	7	9.9	*Litoria aurea* (7)
Hafnia alvei^	5	7.0	*Litoria aurea* (5)
Arcanobacterium haemolyticum^	5	7.0	*Litoria aurea* (5)
Proteus mirabilis^*	4	5.6	*Litoria aurea* (4)
Klebsiella oxytoca^	4	5.6	*Litoria aurea* (4)
Citrobacter sp.^	4	5.6	*Litoria aurea* (4)
*Acinetobacter haemolyticus**	4	5.6	*Litoria aurea* (4)
Citrobacter freundii^*	3	4.2	*Litoria aurea* (1), *Xenopus laevis* (2)
Chryseobacterium meningosepticum^*†	3	4.2	*Litoria raniformis* (3)
Brevibacterium casei	3	4.2	*Litoria aurea* (3)
Aerococcus viridans	3	4.2	*Xenopus laevis* (3)
Acinetobacter sp.^	3	4.2	*Litoria aurea* (3)
Acinetobacter lwoffi	3	4.2	*Litoria aurea* (3)
Stenatrophomonas maltophilia ^*	2	2.8	*Litoria aurea* (2)
Providencia rettgeri^	2	2.8	*Litoria aurea* (1), *Litoria raniformis* (1)
*Flavobacterium indologenes**	2	2.8	*Litoria raniformis* (1), *Xenopus laevis* (1)
Aeromonas hydrophila^*†	2	2.8	*Litoria aurea* (1), *Xenopus laevis* (1)
Pseudomonas sp†	1	1.4	*Litoria raniformis* (1)
Pseudomonas fluorescens ^*†	1	1.4	*Litoria aurea* (1)
Pseudomonas aeruginosa†	1	1.4	*Litoria aurea* (1)
Oerskovia sp.	1	1.4	*Leiopelma archeyi* (1)
Morganella morganii^	1	1.4	*Litoria aurea* (1)
Flavobacterium breve^	1	1.4	*Leiopelma archeyi* (1)
Escherichia coli†	1	1.4	*Xenopus laevis* (1)
Enterobacter agglomerans†	1	1.4	*Litoria aurea* (1)
Comamonas testosteroni	1	1.4	*Leiopelma archeyi* (1)
Chryseobacterium pseudotuberculosis	1	1.4	*Litoria aurea* (1)
Aeromonas trota	1	1.4	*Xenopus laevis* (1)
TOTAL	81		

Table 5.2 Bacteria cultured from non-systemic sites of 47 frogs and their incidence. The sample size for each frog species is given in parentheses. Bacterial species marked with ^ were found in both systemic and non-systemic sites. Species marked with * are known amphibian pathogens, † species are known or suspected to have antifungal properties.

Bacteria	Isolates	% Frogs	Frog Species
Corynebacteria urealyticum^	14	29.8	*Litoria aurea (*14)
Hafnia alvei^	7	14.9	*Litoria aurea* (6)*, Leiopelma archeyi* (1)
Proteus mirabilis^*	6	12.8	*Litoria aurea* (6)
Gram positive unknown	4	8.5	*Litoria aurea* (4)
Klebsiella oxytoca^	4	8.5	*Litoria aurea* (3)*, Leiopelma archeyi* (1)
Providencia rettgeri^	4	8.5	*Litoria aurea* (4)
Arcanobacterium haemolyticum^	3	6.4	*Litoria aurea* (3)
Citrobacter sp.	3	6.4	*Litoria aurea* (3)
Proteus sp.	3	6.4	*Litoria aurea* (1)*, Litoria raniformis* (1)*, Leiopelma archeyi* (1)
Acinetobacter sp.	2	4.3	*Litoria aurea* (2)
Citrobacter freundii^*	2	4.3	*Litoria raniformis* (2)
Enteric unknown	2	4.3	*Litoria aurea* (2)
Enterobacteriaceae sakazakii	2	4.3	*Leiopelma archeyi* (1)*, Leiopelma hochstetteri* (1)
Enterococcus avium	2	4.3	*Leiopelma archeyi* (1)*, Litoria raniformis* (1)
Flavobacterium breve^	2	4.3	*Leiopelma archeyi* (1)*, Leiopelma hochstetteri* (1)
Morganella morganii^	2	4.3	*Litoria aurea* (2)
Myroides odoratus^	2	4.3	*Litoria aurea* (2)
*Serratia liquefaciens**†	2	4.3	*Leiopelma archeyi* (2)
Aeromonas hydrophila^*†	1	2.1	*Litoria aurea* (1)
*Alcaligenes faecalis**	1	2.1	*Leiopelma archeyi* (1)
Corynebacterium aquaticum	1	2.1	*Litoria raniformis* (1)
Chryseobacterium meningosepticum^*†	1	2.1	*Litoria raniformis* (1)
Gram negative rod	1	2.1	*Litoria aurea* (1)
Klebsiella pneumoniae	1	2.1	*Litoria aurea* (1)
Non-pathogenic coagulase	1	2.1	*Litoria aurea* (1)
Plesiomonas sp.	1	2.1	*Leiopelma archeyi* (1)
*Proteus vulgaris**	1	2.1	*Litoria aurea* (1)
Providencia alcalifaciens	1	2.1	*Leiopelma archeyi* (1)
Pseudomonas fluoresens^*†	1	2.1	*Litoria aurea* (1)
Serratia marcesens†	1	2.1	*Litoria raniformis* (1)
Serratia sp.	1	2.1	*Litoria aurea* (1)
Sphingomonas multivorum	1	2.1	*Litoria raniformis* (1)
Stenatrophomonas maltophilia^*†	1	2.1	*Litoria aurea* (1)
TOTAL	81		

Table 5.3 A summary of bacteria isolated from systemic sites, their clinical importance and known hosts

Bacteria	Known hosts		Reference
Acinetobacter haemolyticus	Tiger salamander	*Ambystoma tigrinum*	Worthylake and Hovingh(1989)
	Human: respiratory and urinary tracts, wounds, blood	*Homo sapiens*	von Graevenitz (1995)
Acinetobacter lwoffii	Loggerhead turtle	*Caretta caretta*	Wiles and Rand (1987)
	Orangutan	*Pongo pygmaeus*	Iverson and Connelly (1981)
	Human: respiratory and urinary tracts, wounds, blood	*Homo sapiens*	von Graevenitz (1995)
Acinetobacter sp.	Human: respiratory and urinary tracts, wounds, blood	*Homo sapiens*	von Graevenitz (1995)
Aerococcus viridans	Loggerhead turtle	*Caretta caretta*	Torrent *et al.* (2002)
	American lobster	*Homarus americanus*	Johnson *et al.* (1981)
	Human: rare secondary infection, occasionally linked to endocarditus and bacteremia	*Homo sapiens*	Rouff (1995)
Aeromonas hydrophila	Mountain yellow-legged frog	*Rana muscosa*	Bradford (1991)
	Common frog	*Rana temporaria*	Cunningham *et al.* (1996)
	Wood frog	*Rana sylvatica*	Nyman (1986)
	Many other amphibians and reptiles		Shotts (1984), Taylor *et al.* (2001)
Arcanobacterium haemolyticum	Human: wound, pharagitis, septicemia, endocarditus, osteomyelitis	*Homo sapiens*	Clarridge and Spiegel (1995)
Brevibacterium casei	Human: septicemia in immunocompromised patients	*Homo sapiens*	Clarridge and Spiegel (1995)
Chryseobacterium meningosepticum	South African clawed frog	*Xenopus laevis*	Green *et al.* (1999)
	Bullfrog	*Rana catesbeiana*	Mauel *et al.* (2002)
	Immunocompromised leopard frogs	*Rana pipiens*	Hayes *et al.* (2006)
	Barbour's map turtle	*Graptemys barbouri*	Jacobson *et al.* (1989)
	Human: neonatal meningitis, septicemia	*Homo sapiens*	von Graevenitz (1995)
Citrobacter freundii	Bullfrog	*Rana catesbeiana*	Mauel *et al.* (2002)
	Leopard frog	*Rana pipiens*	reviewed in Taylor *et al.* (2001)
		Ambystoma tridactyla	
	Fire-bellied toad	*Bombina orientalis*	
	Soft-shelled turtle	*Trionyx sinensis*	Wu and Xue (2003)
	Green sea turtle	*Chelonia mydas*	Raidal *et al.* (1989)
	Patas monkey	*Erythrocebus patas*	Ocholi *et al.* (1989)
	Human: gastrointestinal infection, sepsis, numerous other tissues	*Homo sapiens*	Gilchrist (1995)
Corynebacterium urealyticum	Human: alkaline-encrusted cystitus, urinary tract struvite calculi, endocarditis, wound, other.	*Homo sapiens*	Clarridge and Spiegel (1995)
Enterobacter agglomerans	Mountain yellow-legged frog	*Rana muscosa*	Bradford (1991)
	Human: neonatal meningitis, sepsis	*Homo sapiens*	Gilchrist (1995)
Escherichia coli	Green sea turtle	*Chelonia mydas*	Raidal *et al.* (1998)
	Human: urinary tract, intestinal, extraintestinal	*Homo sapiens*	Gray (1995)
Flavobacterium breve	Human: rare	*Homo sapiens*	von Graevenitz (1995)
Flavobacterium indologenes	Leopard frog	*Rana pipiens*	Olson *et al.* (1992)
	Human: rare	*Homo sapiens*	von Graevenitz (1995)
Flavobacterium oderatum	Human: rare	*Homo sapiens*	von Graevenitz (1995)
Hafnia alvei	Cherry salmon	*Oncorhynchus masou*	Teshima *et al.* (1992)
Morganella morganii	Human: urinary tract, extraintestinal, chorioamnionitis, sepsis	*Homo sapiens*	Gilchrist (1995)

Oerskovia sp.	Human: bacteremia, peritonitis, endophthalmitis, endocarditus, meningitis, pyonephrosis	*Homo sapiens*	Beaman *et al.* (1995)
Proteus mirabilis	Human: urinary tract, extraintestinal	*Homo sapiens*	Gilchrist (1995)
Providencia rettgeri	Arizona ridgenose rattlesnakes	*Crotalus willardi*	Ramsay *et al.* (2002)
	Human: urinary tract, extraintestinal	*Homo sapiens*	Gilchrist (1995)
Pseudomonas aeruginosa	Leopard frog	*Rana pipiens*	Brodkin *et al.* (1992)
	Green anole	*Anolis carolinensis*	Jacobson (1984)
	Desert iguana	*Dipsosaurus dorsalis*	
	Blue-tongued skink	*Tiliqua gigas*	
	Trans-pecos rat snake	*Bogertophis subocularis*	
	Indian python	*Python molurus molurus*	
	Ball python	*Python regius*	
	Reticulated python	*Python reticulatus*	
	Gopher tortoise	*Gopherus polyphemus*	
	Human: many	*Homo sapiens*	Gilligan (1995)
Pseudomonas fluorescens	Boa constrictor	*Constrictor constrictor*	Jacobson (1984)
	Burmese python	*Python m. bivittatus*	
	Ball python	*Python regius*	
	Gopher tortoise	*Gopherus polyphemus*	
		Deirochelys reticularia	
	Human: rare	*Homo sapiens*	Gilligan (1995)
Stenatrophomonas maltophilia	Bullfrog	*Rana catesbeiana*	Mauel *et al.* (2002)
	Boa constrictor	*Constrictor constrictor*	Jacobson (1984)
	Human: sepsis, pneumonia, wound	*Homo sapiens*	von Graevenitz (1995)

Table 5.4 Bacteria with known antifungal attributes characterized by chitinase genes

Bacteria	Reference	GenBank Accession Number
Aeromonas hydrophila	Hussein and Hatai 2001	AF251793
Escherichia coli		NC_004431
Pantoea agglomerans	Chernin *et. al* 1995	
Pseudomonas aeruginosa		AF279793, NZ_AABQ07000002
Pseudomonas fluorescens	Reviewed in Whipps 2001	NZ_AAAT03000001
Serratia liquefaciens		AF399871
Serratia marcesens		AF454462 - AF454464
Stenotrophomonas maltophilia	Giesler and Yuen, 1998	AF047411

Discussion

A number of bacteria of clinical interest were isolated both from systemic and non-systemic sites. Of the systemic isolates, *Aeromonas hydrophila*,

Aeromonas trota, *Acinetobacter haemolyticus*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Enterobacter agglomerans*, *Flavobacterium indologenes*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Stenatrophomonas maltophilia* have been associated with amphibian disease.

Aeromonas hydrophila has long been known to be an amphibian pathogen (Frye 1985; Rigney *et al.* 1978; Russell 1898; Shotts 1984), and is commonly associated with the condition known as 'red-leg'. *Aeromonas hydrophila* was considered the causative agent in a number of local population dieoffs including mountain yellow-legged frogs (*Rana muscosa*; Bradford 1991) and wood frogs (*Rana sylvatica*; Nyman 1986). *Aeromonas hydrophila* is generally thought to be an opportunistic pathogen (Cunningham *et al.* 1996) secondary to *B. dendrobatidis* and iridoviruses.

Among the less well known bacteria of amphibian importance, *Chryseobacterium meningosepticum* was responsible for a die-off in a captive population of African clawed frogs (*Xenopus laevis*) (Green *et al.* 1999), and also has been found in bullfrogs (*Rana catesbeiana*) exhibiting 'red-leg' symptoms (Mauel *et al.* 2002). *Citrobacter freundii, Enterobacter agglomerans*, *Flavobacterium indologenes*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* are bacteria that have been associated with amphibian disease and population die-offs in rare cases (Bradford 1991; Green *et al.* 1999; Mauel *et al.* 2002; Olson *et al.* 1992; Taylor *et al.* 2001). I did not consistently culture these bacteria from dead frogs. The most

commonly isolated bacterium, *Corynebacterium urealyticum*, found systemically in over 15% of frogs, has not been described as an amphibian pathogen, although it is an opportunistic pathogen of humans (Clarridge & Spiegel 1995).

Most commonly, the bacteria described here are opportunistic secondary pathogens that are common in the environment. In animals that are immunosuppressed, these relatively benign bacteria have the potential to become pathogenic and can cause mortality. Conversely, bacteria also might protect amphibians from disease. A number of bacteria found on amphibians inhibit the growth of *B. dendrobatidis* (Harris *et al.*, 2006) and *Saprolegnia* sp. (Bly *et al.* 1997; Hatai & Willoughby 1988) a fungus that infects amphibian eggs (Kiesecker & Blaustein 2001; Kiesecker *et al.* 2001). Although bacteria are considered secondary, they remain important to amphibian disease as pathogens and potentially as beneficial bacteria or probiotics.

Of the 29 species of bacteria found in systemic sites, almost 25% of them are known to inhibit, or have the potential to inhibit fungi, and if present on live animals could confer probiotic benefits. *Pseudomonas fluorescens* is a well known fungal antagonist, and is widely used for the bio-control of various fungal pathogens of crops (Whipps 2001). *Pseudomonas fluorescens* also inhibits *Saprolegnia* sp. that are pathogenic to fish and amphibian eggs (Bly *et al.* 1997; Hatai & Willoughby 1988). Members of the genera *Aeromonas*, *Pseudomonas*, *Acinetobacter*, and *Chryseobacterium* are known to be antagonists of a variety of fungi (Chernin *et al.* 1995; R.N. Harris personal

communication ; Hussein & Hatai 2001). Many of the bacteria I found in and on amphibians produce chitinase, an antifungal enzyme, or have chitinase genes, and may exhibit antifungal properties although they have not been directly tested. Other antifungal agents include protease, β-glucanase, iron competing siderophores and antibiotics (reviewed in Whipps 2001). My assessment of antifungal properties based on chitinase presence is conservative as it is limited by the sequences submitted to GenBank, this underestimates the presence of this enzyme among these bacteria.

In this study, I did not consistently find any species of systemic bacteria associated with mortality. Nor did the amphibian chytrid fungus appear responsible for the deaths I observed. As I did not test for viral infections, I am unable to determine viral involvement in morbidity or mortality. This study however, provides an inventory of the bacteria that are associated with amphibians that can be tested for pathogenic or protective qualities.

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CHAPTER 6: GENERAL DISCUSSION

In this thesis I sought to identify some of the factors that predispose amphibians to disease. While amphibian declines are often attributed to infectious agents, especially the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*), it is unclear why amphibians are now susceptible to infection. To help determine these factors, I manipulated the ecological conditions and the genetic constitution of *Xenopus laevis* tadpoles and challenged them with either a model pathogen *Aeromonas hydrophila* or water preconditioned by frogs.

Environmental stressors can increase amphibians' susceptibility to disease, although few studies have directly addressed this. In Chapter 2, I found that many of the ecological factors (crowding, kin composition, habitat complexity) that affect larval growth in other amphibian species also affect *X. laevis* larval growth, but not always in the same fashion. In *Rana pipiens* tadpoles, increasing habitat complexity can ameliorate the growth inhibition of crowding (John & Fenster 1975). While crowding severely reduced *X. laevis* tadpole growth increased habitat complexity did not help crowded *X. laevis* tadpoles grow larger. *Xenopus laevis* tadpoles reared only with their siblings were larger and less variable in size than those reared with unrelated tadpoles (mixed sibling groups), as found in previous studies in tadpoles of other species (Jasieñski 1988; Pakkasmaa & Aikio 2003; Pakkasmaa & Laurila 2004). Bacterial exposure significantly reduced tadpole growth and survival. This growth inhibition was strongest in tadpoles reared in the mixed sibling groups. My finding that groups of tadpoles in the mixed kin condition were more size variable and more susceptible to this pathogen suggests that there was greater competition between

tadpoles in the mixed kin condition than in the sibling groups. Competition and exposure to unrelated individuals can increase physiological stress and may have inhibited the resistance of these tadpoles to the bacterial challenge.

In Chapter 4, I found evidence of another mechanism that may help to explain this pattern of increased susceptibility to disease when among unrelated individuals. In this chapter, I found that tadpoles exposed to water preconditioned by more MHC-different frogs developed more rapidly and were more likely to die than those exposed to water preconditioned by more similar animals. In Chapter 2, tadpoles reared with unrelated individuals may have been exposed to pathogens that were particularly harmful to them, or may have been more severely affected by the *A. hydrophila* that had already infected their unrelated tank-mates. Indeed, there may have been a greater diversity of *A. hydrophila* variants in the mixed sibling groups than in the pure sibling groups, potentially increasing the chance of infection.

I also investigated whether a tadpole's MHC influenced its resistance to disease. MHC based susceptibility and resistance have been documented in many taxa. The only study of MHC based disease resistance in amphibians found that *X. laevis* with the *f* haplotype were resistant and those with the *j* haplotype were susceptible to frog virus 3 (Gantress *et al.* 2003). In Chapter 3, I showed MHC based resistance and susceptibility to a bacterial pathogen, using subjects with some of the same haplotypes as Gantress *et al.* (2003). I found that, when exposed to this pathogen, tadpoles with the *f* or *j* haplotypes were less likely to die than tadpoles with the *r* or *g* haplotypes. Furthermore, tadpoles that were heterozygous at the MHC with both susceptible and resistant haplotypes experienced intermediate mortality and growth

inhibition when challenged with *A. hydrophila*. These are the first results that demonstrate an MHC based resistance or susceptibility to an extracellular pathogen in amphibians.

In Chapter 4, I examined the role of the MHC in disease under more natural conditions. I exposed tadpoles of known MHC genotypes to water preconditioned by adults with different MHC genotypes (sharing 0, 1, or 2 haplotypes). Since larval *X. laevis* share the same environment with adults there is the potential for the vertical transmission of pathogens. Furthermore, adults frequently cannibalise tadpoles (Measey 1998; Parker *et al.* 1947), which, in turn, makes vertical transmission of pathogens even more likely (Pfennig *et al.* 1998). I found that tadpoles exposed to water from MHC-dissimilar animals developed more rapidly, yet without increased growth, and were more likely to die than those exposed to water from MHC-similar animals. I further showed that there appears to be an optimal difference between the tadpoles' and the donors' MHC where tadpoles may be sufficiently different to the donor to avoid its locally adapted pathogens, and sufficiently similar not to be exposed to especially virulent foreign pathogens.

In Chapters 2 and 3, I described differences in survival and growth of tadpoles from the same parents but from clutches of eggs laid at different times. In Chapter 2, I found that tadpoles from the same parents that bred a week later than their older siblings were significantly smaller and more likely to die than their older siblings. In Chapter 3, I found that tadpoles from eggs laid later in the evening were both larger, and were less likely to die than their siblings that were from eggs laid 6 hours earlier. While these results are interesting, it is difficult to assess their significance given the

artificial breeding regime I used. Intra- and interclutch differences have been described in several amphibian species (Crump 1984; Dziminski & Alford 2005; Dziminski & Roberts 2006; Kaplan 1980, 1989, 1992; Parichy & Kaplan 1992, 1995; Smith 1999; Tejedo & Reques 1992) but not for *X. laevis*. If this pattern of differential maternal investment in eggs exists in more natural conditions it might provide insight into the mechanism of intraclutch variation described in other species. Differential investment in eggs might be beneficial as females could vary the amount of investment in eggs to 'hedge their bets' on the environmental stability of the breeding ponds (Crump 1981; Crump 1984; Kaplan & Cooper 1984). Tadpoles from poorly provisioned eggs might survive when pond conditions are favourable but would die when conditions are adverse. Females may otherwise lay poorer quality eggs early in a breeding attempt to satiate oophagous predators thereby protecting her higher quality offspring, or as a mechanism of female choice. Females may release their best quality eggs only to males that are capable of extended copulation: a costly activity in a competitive environment, thereby assuring that high quality males fertilize her best quality eggs. These results are also important because of *X. laevis*' use as a laboratory animal. *Xenopus* is used extensively in molecular and developmental biology and often researchers try to get large numbers of healthy eggs and tadpoles. To do so researchers may repeatedly breed females, as it is easy to obtain a series of reasonably large clutches. In doing so, researchers may be unknowingly adding considerable noise to their data, as there may be fundamental differences between tadpoles from different breedings, and within breedings. Further studies that examine the differences in survival and growth between tadpoles from early and late eggs within a single clutch without the added factor of multiple breedings, would further clarify whether this finding is a laboratory artefact or an interesting biological pattern

worthy of further study. Sequentially moving amplectant frogs to different tanks during mating would easily test this. If these intra- and interclutch differences in growth and survival are repeatable a number of further experiments could examine the hypotheses outlined above. Comparing the intraclutch variation of clutches laid in ponds with differing predation risk could test the predator satiation hypothesis. This hypothesis suggests that females breeding in ponds with more oophagous predators should produce more poor quality eggs early in the breeding attempt. Presenting eggs sequentially to oophagous predators could also determine whether they can be satiated, and how many eggs must be sacrificed. The female choice hypothesis could be tested by manipulating the quality of males and the competition between males during breeding. If some males successfully fertilise a greater proportion of the later eggs than the earlier eggs the female choice hypothesis would be supported. The fertilization success of different males would need to be compared with an artificial breeding control where equal quantities of each males' sperm is presented to the eggs to control for differences in sperm viability, motility and sperm-egg compatibility.

In Chapter 5 I presented a catalogue of bacteria I isolated from systemic (heart, muscle, abdominal cavity) and non-systemic (skin and gut) sites in five species of frogs. While some of the pathogens I described in Chapter 5 have been previously identified as amphibian pathogens, many have not. I identified a number of bacteria that are known to have, or are suspected to have, antifungal properties. This inventory of bacteria will provide a list of bacteria of note for both pathogenic and probiotic purposes. These bacteria might prevent infection by fungal pathogens, as a number of bacteria have recently been found to inhibit the growth of the amphibian

chytrid fungus *in vitro* (Harris *et al.* 2006). I proposed eight bacterial species that have the potential to prevent the growth of the amphibian chytrid fungus based on the presence of chitinase genes or previously documented antifungal properties.

In total, my work described a number of factors that can affect amphibians' ability to resist disease. Not only have I shown that the genetic constitution of an individual, specifically in terms of the MHC, affected the impact of a disease, but so too did its social and ecological conditions, including the level of crowding, the kin composition of its group and the specific microbial challenges of its immediate environment. Furthermore I showed that many of the factors linked to tadpole growth and development that are well described in other amphibians also affected *Xenopus* tadpoles.

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